Endophytes of *Pseudowintera colorata* (horopito)

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By

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Pseudowintera colorata (horopito) commonly known as New Zealand pepper tree is a native medicinal plant, known for its antimicrobial properties. International studies have demonstrated that endophytes of medicinal plants play key roles in maintaining plant health, tolerance to biotic and abiotic stresses and production of secondary metabolites. However, there have been no studies on the endophytes of *P. colorata*. The objectives of this study were to i) investigate the community structure of the endophytic bacteria, Actinobacteria and fungi in the tissues of *P. colorata* and identify members of the core endomicrobiome, ii) investigate the bioactive potential of the culturable endophytic bacteria, Actinobacteria and fungi, iii) investigate the influence of selected members on the growth and chemistry of *P. colorata in vitro* and *in vivo*.

The endophytic communities in *P. colorata* were characterized by denaturing gradient gel electrophoresis (DGGE) and Illumina MiSeq. Plants from ten sites across New Zealand were analysed by DGGE and it was revealed that tissue type was the main factor influencing the endophytic communities in *P. colorata* (PERMANOVA, P=0.001). Richness of Actinobacteria, Betaproteobacteria and fungi was higher in stems compared to leaves and roots. For a subset of three sites, the interaction of plant location with maturity influenced the microbial communities across all groups analysed except for Alphaproteobacteria and total fungi (PERMANOVA, P=0.226 and P=0.164 respectively). Using Illumina MiSeq for analysing the bacterial communities from ten sites, it was confirmed that tissue type affected the bacterial communities in *P. colorata*. Illumina data revealed that Gammaproteobacteria was the most abundant class (89.1%) followed by Alphaproteobacteria (10%). In addition, two OTUs belonging to *Pseudomonas* were identified as members of *P. colorata* core endomicrobiome.

A total of 350 endophytic bacteria, 200 endophytic fungi and nine endophytic Actinobacteria were recovered from *P. colorata* plants from ten sites across New Zealand. The majority of

endophytic bacteria were isolated from the stem (57.1%, n=200), followed by roots (37.1%, n=130) and leaves (5.7%, n=20). Eleven endophytic bacteria showed strong antagonistic activity against four phytopathogenic fungi *Neofusicoccum luteum*, *N. parvum*, *Neonectria ditissima*, *Ilyonectria liriodendri* and four endophytic bacteria were active against bacterial pathogens *Pectobacterium atrosepticum*, *P. brasiliensis*, *Staphylococcus aureus*, *Escherichia coli*. In addition, endophytic bacteria also produced siderophores on chrom-azurol S agar (CAS). Based on 16S rRNA gene, the endophytic bacteria were identified as members of genera *Bacillus*, *Pseudomonas*, and *Pantoea*. The endophytic fungi *Pezicula* sp. PRY2BA2, *Metarhizium* sp. PR1SB1 were active against all the phytopathogenic fungi tested. Six endophytic fungi showed high activity against *Candida albicans in vitro*.

A total of nine endophytic Actinobacteria were recovered onto selective agar. Sequencing the 16S rRNA gene revealed that the culturable members belonged to genera *Streptomyces*, *Micromonospora*, *Nocardia*, *Nakamurella* and *Microlunatus*. Major bands (n=20) from DGGE gels were sequenced and were identified as uncultured bacteria, *Streptomyces* sp. and *Angustibacter peucedani*. *Nocardia* sp. TP1BA1B and *Streptomyces* sp. UKCW/B solubilized phosphate in tricalcium phosphate agar (TCP) and secreted siderophores. This is the first study to identify Actinobacteria communities in *P. colorata* and to examine the functional traits of cultured representatives.

The effect of endophytes displaying *in* vitro activity was examined by reintroduction as a soil drench. *Bacillus* sp. TP1LA1B and *Nocardia* sp. TP1BA1B in addition to increasing the shoot height, also significantly increased the shoot, root biomass and the number of internodes of *P. colorata* seedlings compared to the control (P=0.016, P <0.001, P=0.007 and P <0.001 respectively). This is the first study to investigate the influence of endophytes on the growth of *P. colorata*.

Overall, this study revealed the community structure of endophytic bacteria, Actinobacteria and fungi in *P. colorata* for the first time and is one of the only two studies on native plants in New Zealand. Members of the endomicrobiome displayed *in vitro* activity as measured by antimicrobial and nutrient mobilisation assays. The endophytes were able to influence host plant growth when applied as soil drenches and some were able to recolonize the host endophytically demonstrating a route from soil to root. This study indicated that *P. colorata* harbours unique endophytes which have a key role in the ecology of the plant.

Keywords: Endophytes, Microbial Ecology, Medicinal Plant, *Pseudowintera colorata*, horopito, biological control, DGGE, NGS, Illumina MiSeq, Plant growth promotion, Endophytic Actinobacteria, biotransformation.

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Chapter 1

Literature Review

1.1 Endophytes

Endophytes are microorganisms that inhabit host plants for all or part of their life cycle and most endophytes produce a vast array of compounds that are likely involved in the host-endophyte relationship (Schutz, 2001). Endophytes constitute a broad range of organisms including bacteria, algae and fungi (Schulz and Boyle, 2005). Though the strict definition of the term endophyte has been long debated, the following definition of endophytes is widely accepted: "*Endophytes are microbes that for all or part of their lifecycle, invade the tissues of living plants and cause unapparent and asymptomatic infections entirely within plant tissues, but cause no symptoms of disease*" (Wilson, 1995).

1.1.1 Endophytic fungi

Endophytic fungi are a highly diverse group. The most well recognized group is the Glomeromycota which form arbuscules in plants and are commonly referred to as arbuscular mycorrhizal fungi (AMF). The AMF are distributed widely and form functional associations with the roots of the majority of plant species worldwide (Berruti *et al.,* 2015). These fungi are not the subject of this thesis and are not described further. The majority of other endophytic fungi have been identified as ascomycetes and many of these fungi lack a known teleomorphic state (Carroll, 1988). Far less is known about this group although it is clear they can have significant impacts on plant physiology and ecological fitness (Porras-Alfaro and Bayman, 2011).

Over the past two decades, there have been at least two different ways of grouping fungal endophytes: the 2-class classification and 4-class classification system (White 1988; Rodriguez *et al*., 2008). According to the 2-class system suggested by White, (1988), emphasis was placed on the transmission mode in particular and the fungi were classified as belonging to Clavicipitaceae (clavicipitaceous endophytes) or not (non-clavicipitaceous endophytes). There are two modes of transmission, vertical and horizontal. Vertical transmission refers to the transmission of endophytes from host plant to the offspring via seeds whereas horizontal transmission refers to the transmission of endophytes via

environment through wounding or natural entry sites of leaves, etc. In the 4-class classification system of fungal endophytes as suggested by Rodriguez *et al.* (2009) the highly diverse nature of the non-clavicipitaceous endophytes was also taken into account. This is the system used in this thesis and will be described briefly here.

Rodriguez classification system:

Class 1 endophytes consist of the clavicipitaceous endophytes and are characterized by vertical transmission, with adult plants passing on fungi to offspring via seed. They have a narrow host range and colonization is limited to the shoot, stem and rhizome. Many members of the class 1 endophytes are thought to enhance resistance to invertebrate herbivory; however, there have been inconsistencies with this theory due to the fact that the enhanced function might the result of interactions between specific host/fungal genotypes and not broadly attributable to a fungal species (Clay, 1990; Faeth *et al.,* 2006).

Class 2 endophytes include a diversity of species of the subkingdom Dikarya. Most of the members of Class 2 belong to Ascomycota, but a minority of Basidiomycota members have also been reported. The mode of transmission of the endophytic fungi to the host plant is either horizontal or vertical. When the spores infect a new host through the surface of the plant it is described as horizontal transfer, but when the endophytic fungus infects the seeds it is referred to as vertical transfer (Rodriguez *et al*., 2009). Class 2 endophytes exhibit both vertical and horizontal modes of transmission; they extensively colonize within the entire plant including the roots, stems and leaves. Although the biodiversity of class 2 endophytes per plant is generally low, the host range is broad and they are also attributed with conferring the host plants with habitat adapted benefits like salt tolerance in plants colonizing coastal beaches (Rodriguez *et al*., 2008). Studies by Macia'-Vicente *et al.* (2008 a, b) on Mediterranean plants revealed that *Phoma* spp. were common root endophytes and conferred fitness benefits to the host plants. Studies have also shown that some class 2 endophytes may speed up host defences when exposed to pathogens through the production of some bioactive agents (Redman *et al*., 1999). Schulz *et al.* (1999) demonstrated that that the ability of the endophytic fungi *Fusarium oxysporum* and *Cryptosporiopsis* sp. to protect barley and larch, respectively against phytopathogens were correlated to the increased concentrations of phenolic metabolites.

Class 3 endophytes are transmitted horizontally and have a broad host range; low colonization in plants and infection is restricted to shoots. However, the ecological roles of these fungi are largely unknown. Members of the Basidiomycota belonging to the Agaricomycotina, Pucciniomycotina, and Ustilaginomycotina are some of the known examples of Class 3 endophytes.

Class 4 endophytes are commonly referred to as dark septate endophytes (DSE) and are characterized by the presence of melanized hyphae. They occur only in the root and are horizontally transmitted; little is known about their ecology, except that their distribution seems to be almost cosmopolitan and they are especially common in high-stressed habitats (Rodriguez *et al*., 2008; Hardoim *et al*., 2015).

1.1.2 Endophytic Bacteria

Endophytic bacteria are referred to as those that colonize the plant interior and can be detected within the tissues of apparently healthy plant hosts (Schulz and Boyle, 2005). Most of these endophytes colonize different compartments of the plant apoplast, including the intercellular spaces of the cell walls and xylem vessels. Some of them are able to colonize reproductive organs of plants, e.g. flowers, fruits and seeds without causing any substantial morphological changes like root-nodule symbionts do (Hallmann *et al*., 1997; Hallmann 2001).

There are well known examples of endophytic bacteria such as rhizobia, which fix atmospheric nitrogen and make it available to their leguminous hosts (Bhattacharjee *et al*., 2008). The interaction between rhizobia and the host plant is well-defined involving complex signalling, which results in the root tissue being differentiated into nodules. However, the interaction and function of other endophytic bacteria is not fully understood. More than 200 bacterial genera from 16 phyla including both culturable and unculturable bacteria belonging to Acidobacteria, Actinobacteria, Aquificae, Bacteroidetes, Cholorobi, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Firmicutes, Fusobacteria, Gemmatimonadetes, Nitrospira, Planctomycetes, Proteobacteria, Spirochaetes and Verrucomicrobiae have been reported as endophytes isolated from surface sterilized plant material (Berg and Hallmann, 2006; Sessitsch *et al.,* 2012). However, the most predominant and studied endophytes belong to three major phyla viz. Actinobacteria, Proteobacteria

and Firmicutes; including members of *Bacillus* (Deng *et al*., 2011), *Burkholderia* (Weilharter *et al*., 2011), *Herbaspirillum* (Pedrosa *et al*. 2011), *Pseudomonas* (Taghavi *et al*., 2009), *Serratia* (Taghavi *et al*., 2009), *Stenotrophomonas* (Ryan *et al.,* 2009) and *Streptomyces* (Suzuki *et al*., 2005).

Species of these genera are ubiquitous in the rhizosphere, which represents the main source of endophytic colonizers (Berg and Hallman, 2006). Other possible sources of endophytes include the phyllosphere, the anthosphere and seeds (Compant *et al*., 2010). Endophytes can enter the plants through natural wounds such as emergence of lateral roots and can also enter the tissues actively through the production of hydrolytic enzymes (Lodewyckx *et al.,* 2002). In addition, natural openings such as stomata, lenticels and hydathodes may also act as entry sites for phyllosphere bacteria to penetrate and colonize host plants (Hallmann, 2001).

Endophytic bacteria also do not cause any disease symptoms, in contrast to phytopathogens. Many endophytic bacteria possess several plant-beneficial traits *in vitro* but only a fraction exhibit them *in vivo* and of these only a small number of endophytes prove to be very effective plant-growth promoting and/or biocontrol agents under agricultural conditions (Scherwinski *et al*., 2008). A study by Liu *et al*. (2016) on the Chinese medicinal plant *Ferula songorica* revealed that the plant was a rich reservoir for endophytic bacteria which were capable of growing on nitrogen-free media, solubilizing phosphate and producing enzymes such as protease and cellulase.

1.1.3 Endophytic Actinobacteria

Actinobacteria are an important and ubiquitous group of bacteria that are widely distributed through many ecosystems and play a pivotal role in recycling organic matter (Delavat *et al*., 2012). The interaction between Actinobacteria and plants is ideally symbiotic wherein the Actinobacteria secrete herbicidal and antimicrobial compounds, fix atmospheric nitrogen and protect plants against fungal infections (Boddey and Dobereiner 1995).

Several studies have demonstrated that roots harbour a majority of Actinobacteria compared to leaves and stems (Passari *et al*., 2015; El-Tarabily *et al*., 2009). These studies support the evidence that endophytic Actinobacteria enter the plants through roots. In

addition to entry via roots and natural openings, studies have shown that endophytic Actinobacteria can enter the plant via some specific mechanisms. For example, Trujillo *et al.* (2014) revealed that the genome of an endophytic Actinobacteria *Micromonospora lupine* strain Lupac 08, contained several genes encoding proteins to neutralize oxidative stress mounted by host plants.

Although the interaction between several Actinobacteria and their host plants is not fully understood; many isolates showed beneficial effects and may play an important role in the physiology of the plant (Ulrich *et al*., 2008). A well understood example of endophytic Actinobacteria is *Frankia* spp. which are capable of fixing atmospheric nitrogen and play an important role in plant ecology. Studies have revealed that endophytic Actinobacteria belonging to genera including *Streptomyces*, *Actinomadura*, *Gordonia*, *Microbispora*, *Micromonospora*, *Nocardia*, *Arthrobacter, Rhodococcus* and *Pseudonocardia* are commonly found as endophytes of plants (Taechowisan *et al.,* 2003; Kaewkla and Franco, 2013).

Production of biologically important compounds by endophytic Actinobacteria has been demonstrated by many studies. For example, a study by Salam *et al*. (2017) on the Chinese medicinal plant *Dracaena cochinchinensis* showed that this plant was host to Actinobacteria producing metabolites with antimicrobial, antifungal and cytotoxic activity. Research by Qiu *et al*. (2015) studied the diversity and metabolic activity of Actinobacteria from 13 traditional Chinese medicinal plants and revealed that 15% of the total Actinobacteria (n=80) were active against one indicator test pathogen and in addition, 87.5% and 58.8% of the metabolites of the isolates showed anticancer and anti-diabetic activity, respectively.

1.2 The function of endophytes

After establishing themselves in a plant, endophytes can positively influence plant growth and its responses under stress (Berg, 2009). Endophytes support plant growth and productivity either through direct mechanisms via nutrient uptake, production of plant growth promoting hormones or indirect mechanisms such as tolerance to heavy metals, salinity and protection against plant pathogens (Chen *et al*., 2000; Lodewyckx *et al.,* 2002). Endophytes associated with medicinal plants have been shown to produce several novel

antibiotics (Castillo *et al.,* 2002) and can have biocontrol potential (Miller *et al*., 1998; Tianxing *et al*., 2013). Endophytes can prevent colonisation of phytopathogens by competing for niches, releasing enzymes and antimicrobial compounds (Strobel 2003; Strobel and Daisy 2003; Berg and Hallmann, 2006). In addition, endophytes influence the fitness of the host plant via the production of anti-herbivore alkaloids and enhancing photosynthesis (Sanchez-Azofeifa *et al.,* 2012; Gundel *et al.,* 2012).

1.2.1 Nutrient Uptake

Endophytes mediate plant growth promotion by assisting with nutrient uptake such as solubilizing phosphate and fixing nitrogen (Ryan *et al*., 2008). Some of these processes are done by endophytes with relatively well described associations, such as those between rhizobia and leguminous plants. The phosphate solubilization is normally brought about by the production of organic acids into the soil, which solubilize the phosphate complexes and convert them into ortho-phosphate which is utilized by the plant. Gupta *et al*. (2012) showed that inoculation of *Aloe barbadensis* Miller with the endophyte *Serratia marcescens* increased the soil available P and uptake of phosphate by 184% in comparison to the uninoculated control. Though the ability of bacteria to solubilise P is also well described for rhizosphere colonisers, the advantage of this trait in endophytes *in planta* is not fully understood. A recent study by Oteino *et al.* (2015), demonstrated that endophytic bacteria producing medium to high levels of gluconic acid were able to stimulate the growth of *Pisum sativum* plants grown in soil under limited soluble phosphate conditions.

International research has suggested that the mutual benefit between endophytes and host plants is dependent on the availability of nutrients, particularly nitrogen (N) (Saikonnen *et al*., 2006; Xu et al., 2012). Li *et al.* (2016c) reported that inoculation with the endophyte *Epichloë gansuensis* influenced the shoot biomass of *Achnatherum sibiricum* and this was dependant on the nitrogen supply. AMF are considered as natural biofertilizers and in exchange for photosynthetic products provide hosts with nutrients, help with water uptake and protect against pathogens (Berruti *et al.,* 2015). Several international studies have described AMF associated with medicinal plants globally (Taber and Trappe, 1982; Wei and Wang 1989; Gorsi, 2002). The only published studies demonstrating the association of New Zealand medicinal plant with AMF were in *L. scoparium* (McKenzie *et al*., 2006; Wicaksono, 2016). Wicaksono, (2016) demonstrated

that inoculation of some AMF isolates in *L. scoparium* significantly increased the plant growth in addition to modifying the essential oil composition quantitatively and also altered the community structure of Gammaproteobacteria in rhizosphere and roots.

1.2.2 Protection against phytopathogens

Endophytes can protect plants either directly or indirectly. Biocontrol of phytopathogens can be based on several mechanisms including antibiosis, competition for nutrients and induced systemic resistance (Berg and Hallman, 2006). Direct protection is usually via the production of antimicrobial compounds, for example, Silva *et al*. (2006) demonstrated that the endophytic fungus *Phomopsis cassiae* isolated from *Cassia spectabilis* produced 3,11, 12-trihydroxy cadalene which had strong antifungal activity against *Cladosporium sphaerospermum*. In addition to antimicrobial substances, endophytes can also secrete lytic enzymes against compounds like chitin, proteins, cellulose, hemi-cellulose and DNA (Tripathi *et al*., 2008).

The indirect mechanism of protecting the host against phytopathogens is via the production of compounds such as siderophores. Iron (Fe) is an essential element that is needed for metabolic processes. Though Fe is abundant in soil, most of it is not available at neutral pH. Microorganisms are capable of producing siderophores, which can chelate Fe under Fe-limiting conditions (Mukai *et al*., 2009; Patzer and Braun 2010). The affinity of these siderophores vary and this often causes competition between pathogenic and non-pathogenic microorganisms (Wensing *et al*. 2010). High affinity siderophores produced by some microbes can make Fe unavailable for pathogens, thus resulting in reducing infection (Whipps, 2001). A study by Combès *et al.* (2012) revealed that the endophytic fungus *Paraconiothyrium variabile*, isolated from the host plant *Cephalotaxus harringtonia*, was able to inhibit the growth of common phytopathogens, thus suggesting a role in its host protection.

1.3 Commercial interest in endophytes

In addition to protecting plants from phytopathogens and promoting host growth, endophytes are known to produce several bioactive secondary metabolites that have been used as sources of drugs for treating various diseases and have potential applications in agriculture, medicine and food industries (Strobel and Daisy, 2003). Some endophytes, due

to years of close interaction with their host, can produce the same compounds as the host. For example, Taxol® an anticancer compound produced from the bark of *Taxus brevifolia* is also produced by the endophytic fungi *Taxomyces andreanae* (Stierle *et al.,* 1993). Isolation of *T. andreanae* has allowed for the inoculation of the host to enrich for this substance. Taxol® has since been found to be produced by several other endophytic fungi such as *Metarhizium anisopliae and Pestalotiopsis terminaliae* isolated from *Taxodium distichum* and *Wollemia nobilis* respectively (Li *et al.,* 1996; Strobel *et al.,* 1997; Zhang *et al.,* 2009; Gangadevi and Muthumary, 2009). Podophyllotoxin, known for its anticancer and antiviral properties, is naturally produced by *Podophyllum* spp. which are endangered plants. However, the identification of an endophytic fungus *Trametes hirsute* as an alternative producer of podophyllotoxin has reduced the usage of the plant for production of the compound (Puri *et al.,* 2006).

1.4 Endomicrobiome and functionality analysis

The endomicrobiome is the population of microbes that resides within plant tissues and can confer unique characteristics to the host. Due to its functional significance, exploring the endomicrobiome has garnered a great deal of attention in the field of plant-microbe interactions. This second genome supplied by microorganisms adds to the existing genetic complexity of plants with a multitude of properties with a key influence on plant growth, development and disease resistance (Schutz, 2001).

Analysis of the endomicrobiome can use either culture-dependent or independent techniques. Culture dependent approaches involve the isolation and creation of a library of microbes that can be later tested for bioactivity both *in vitro* and *in vivo*. However, culture dependent methods alone offer limited information as only a small representative number of endophytes are recovered (Jin *et al*., 2014). Due to this drawback, culture independent methods like denaturing gradient gel electrophoresis (DGGE) and next generation sequencing have been used to study the total microbial community (Cleary *et al*., 2012; Yu *et al*., 2015).

1.4.1 Culture independent methods

1.4.1.1 Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is one of the most widely used culture independent molecular techniques. It separates DNA fragments that are of the same length but have different sequences. The principle behind the technique is that DNA fragments are separated based on the decreased electrophoretic mobility of a partially melted double stranded DNA in polyacrylamide gels containing a gradient of DNA denaturants (Muyzer and Smalla, 1998). The microbial population diversity in the sample is represented in DGGE gels as different bands. The small subunit ribosomal RNA like the 16S rRNA is ideal to compare the cellular and molecular evolution because it contains sufficient information for comparison and measurement of close and distant phylogenetic relationships (Sogin *et al*., 1986b). For fungi, the internally transcribed spacer (ITS) region of the rDNA can be used for taxonomy because it is easy to amplify even with low quality samples, shows variation to distinguish among species and availability of sequence databases to identify fungi (Nilsson *et al*., 2008).

Although the 16S rRNA and 18S rRNA genes are most commonly used, other genes used are *nifH* for nitrogen fixing bacteria, *amoA* for ammonia-oxidizing bacteria communities, etc. (Dias *et al*., 2012; Puglisi *et al*., 2012). Using DGGE Nimnoi *et al*. (2010) revealed that the roots of *Aqualaria crassna* from two different provinces in Thailand had different endophytic Actinobacteria communities and that roots had the highest diversity of endophytic Actinobacteria compared to other issues. In addition, Nimnoi *et al.* (2010) revealed that the endophytic Actinobacteria in *A. crassna* produced phytohormones, siderophores which may have a role in host growth. A similar study by Garcias-Bonet *et al*. (2012) used DGGE to analyze the bacterial communities in the marine angiosperm *Posidonia oceanica* and found that root communities significantly differed from rhizomes and leaves. The recent study by Wicaksono *et al.* (2016) used DGGE to analyze the communities of endophytic bacteria in the *L. scoparium* and revealed tissue type as the main factor influencing the communities. DGGE has been proven to be very efficient for analyzing endophytic bacterial communities in plants tissues at a relatively low concentration (10^2 CFU/g fresh plant tissue) (Garbeva *et al*., 2001).

Although widely used, DGGE has some limitations such as over estimating and under estimating the community diversity, and the inability to detect minor components of the microbial community (Mühling *et al*., 2008). The use of specific primers for DGGE may result in the PCR product to be composed mainly of the abundant species, while misrepresenting the other species present in lower numbers. The other disadvantages of DGGE include variations from gel to gel, co-migration of different taxa in the same band, and visualization of only the more abundant taxa (Muyzer *et al*., 1993; Dowd *et al.,* 2008). Resolution related issues can be mitigated by the use of taxon specific primers such as α, β and γ Proteobacteria groups (Mühling *et al.,* 2008).

1.4.1.2 DNA metabarcoding using next generation sequencing technologies

Nucleic acid sequencing is a molecular method for determining the exact order of nucleotides present in a given DNA or RNA molecule (Ayman and Weinbrecht, 2013). The principal approaches which are currently used to assign taxonomy to DNA sequences are DNA metabarcoding and metagenomics. While metabarcoding focuses on the describing the taxonomy of the species present in the sample, metagenomics characterizes the genomes present in an environmental sample, using both a taxonomic and a functional analytical approach (Zepeda Mendoza *et al.,* 2015). Metabarcoding using Illumina MiSeq is a powerful tool to study the endophytic communities in samples.

DGGE analysis is being displaced by these new sequencing technologies which are able to provide a deeper observation by detecting more species with greater accuracy (Yu *et al*., 2015; Qin *et al*., 2016). These new platforms perform massively parallel sequencing, during which millions of fragments of DNA from a single sample are sequenced in unison. Massively parallel sequencing technology facilitates high-throughput sequencing, which allows an entire genome to be sequenced in less than one day. Two of the most commonly used platforms are: the Life Technologies Ion Torrent Personal Genome Machine (PGM) and the Illumina MiSeq/ pyrosequencing (Ayman and Weinbrecht, 2013).

Using Illumina MiSeq, Akinsanya *et al*. (2015) found that *Proteobacteria, Firmicutes, Actinobacteria* and *Bacterioidetes* were the predominant genera in the roots of *Aloe vera*. Using Illumina MiSeq platform, Wicaksono (2016) found that Gammaproteobacteria was the most abundant class in the tissues of *L. scoparium*. Using a metagenomics approach,

Yuan *et al*. (2016) demonstrated that the microbiome of endophytic bacteria associated with on the halotolerant plant *Suaeda salsa* (seepweed) had functional gene categories related to salt stress acclimatization, nutrient solubilisation and competitive root colonization.

One of the drawbacks of molecular techniques is that they cannot differentiate between true endophytic DNA and residual DNA from dead cells and epiphytes. Several studies have demonstrated that using propidium monoazide (PMA), this issue can be mitigated (Nocker *et al*., 2007; Taylor *et al*., 2014; Wicaksono *et al*., 2016). Propidium monoazide is a DNA-intercalating dye, which is able to enter cells with compromised membranes and irreversibly bind to DNA. Nocker *et al.* (2007) demonstrated that PMA treatment significantly suppressed the signals of killed cells and improved the DGGE banding pattern and the intensity of community profiles. A similar study by Wicaksono (2016) also demonstrated that PMA treatment excluded DNA from dead bacteria.

1.4.2 Culture dependent techniques

In order to recover endophytes, studies have employed several different types of media. The nutrient media commonly employed can be broadly classified as minimal media and complex media. Usually for the recovery of endophytic bacteria and fungi, minimal media are employed. Minimal media such as R2A, SNA, M3 and M9 are commercial media and allow the growth of slow growing endophytic bacteria and fungi (Basu *et al*., 2015; Eevers *et al.,* 2015). Though different studies have employed different media, there has been no clear consensus on the best media to isolate endophytic bacteria. Endophytic Actinobacteria are slow growers and have complex nutritional requirements. International studies have used media such as international *Streptomyces* project media (ISP 1, 2 and 4) for the isolation of endophytic Actinobacteria especially *Streptomyces* (Passari *et al*., 2015; Araujo-Melo *et al*., 2017). Other studies have used a combination of multiple media to increase the frequency of isolating rare endophytic Actinobacteria, for example, Kaewkla and Franco (2013) used ten different media to isolate Actinobacteria from four different Australian native trees and isolated a total of 576 endophytic Actinobacteria belonging to genera including *Streptomyces*, *Actinomadura*, *Gordonia*, *Micromonospora*, *Nocardia*, and *Pseudonocardia*.

To conduct rapid screening of culturable endophytes, plate-based assays are routinely used. Using plate-based assays, traits linked to plant growth, nutrient uptake, production of enzymes, secretion of siderophores and protection against pathogens can be carried out. Microbes capable of solubilizing phosphate convert insoluble phosphorus to a soluble form that can influence plant growth under field conditions (Verma *et al.,* 2001). Media containing insoluble inorganic phosphate such as tricalcium phosphate (TCP) are routinely used to screen the ability of endophytic bacteria, Actinobacteria (Frey-Klett *et al*., 2005; Gupta *et al*., 2012). Presence of a clear zone indicates the capability of the test strain to produce organic acids which solubilize phosphate on a TCP plate (Mehta and Nautiyal, 2001). The potential of endophytes as biocontrol agents is commonly assessed by their ability to secrete siderophores and activity against phytopathogens on dual culture assays (Berg *et al*., 2002). Berg *et al.* (2002) used Waksman agar to screen bacteria for antagonism against *Verticillium dahlia.* Chrom-azurol S (CAS) agar is routinely employed to identify bacteria capable of producing siderophores, which is shown by an orange halo zone in CAS agar (Mukai *et al*., 2009; Patzer and Braun 2010).

The effect of endophytes on plant growth is studied by re-inoculation either into a model plant system or a new host plant. Some of the common methods include soil drenching, foliar spray, root dipping, seed inoculation and combination of soil dipping and seed inoculation. A study by Zakria *et al.* (2008) demonstrated higher bacterial densities of *Pantoea* sp. strain 18 and *Enterobacter* sp. strain 35 as endophytes were obtained by root dip method compared to rhizosphere inoculation in cultivated rice. Greenfield *et al.* (2016) demonstrated that using a soil drenching method *Beauveria bassiana* and *Metarhizium anisopliae* endophytically colonized cassava roots for up to seven weeks and found differences in colonization success and plant growth.

1.5 Endophytes of New Zealand plants

Plants used in traditional medicine (Rongoā plants) are recognized for their bioactive properties. As with international examples, New Zealand medicinal plants are likely to host unique endophytes with as yet uncharacterized functions (Araújo *et al*., 2002; Garcias-Bonet *et al*., 2012; Da Silva *et al.*, 2013). Research on endophytes of native New Zealand plants is scarce with a significant knowledge gap with respect to the functional diversity of the endophytes inhabiting these plants. Studies on other medicinal plants internationally

have shown that the endophytes in these plants play a key role in plant growth and health and can have unique properties such as production of bioactive compounds. The only comprehensive study on the endophytic bacterial communities of a medicinal plant in New Zealand was of *L. scoparium* (Wicaksono *et al.,* 2016). Wicaksono *et al.* (2016, 2017a) demonstrated that the endophytic bacteria from *L. scoparium* had a key role in host physiology by enhancing growth and modifying the composition of the essential oils. In addition, they were able to control phytopathogens and were transferrable to other commercial plants such as grapevine.

1.6 *Pseudowintera colorata*

Pseudowintera colorata (Raoul) Dandy, also commonly known as horopito or New Zealand pepper tree is a member of the Winteraceae family (Riley, 1994). Plants of the family Winteraceae are widespread and are present in most parts of the world; however New Zealand has its own endemic genus, *Pseudowintera*, with four species namely *P. colorata, P. axillaris, P. insperata* and *P. traversii*. *Pseudowintera colorata* is a shrub growing to about 3.5 m in height and has a woody trunk and upright branches (Corbett and Grant, 1958). The leaves grow in an alternate fashion and have an upper surface that is matt green or yellowish-green and is often blotched with red, where red is more prominent towards the outer margins in exposed situations (Fig. 1.1). The undersides of the leaves are glaucous to white and often pink-flushed (Allan, 1961). The leaves are characterized by a pungent and peppery taste. The fruits of *P. colorata* are dark red or black, fleshy and usually have about 2 to 3 seeds. It is believed to have several primitive features, which are very similar to the earliest evolving plants. Fossil records have suggested that horopito has been in existence for more than sixty five million years (Webb *et al*., 1990). *Pseudowintera colorata* is distributed across North, South and Stewart Islands and occurs from lowland to montane forest communities. Mildenhall (1980) reported that in New Zealand, the Winteraceae pollen were from the Upper Cretaceous sediments.

Figure 1.1: *Pseudowintera colorata* leaves with red blotched margins

1.6.1 Rongoa Māori and importance of *P. colorata*

The Māori arrived from Polynesia sometime around 1300 A.D. (Wilmshurst *et al*., 2008) and soon became familiar with the native flora of New Zealand. They started finding uses for the endemic plants for tools, building, clothing and medicine (Riley, 1994). They employed 'Rongoa Māori', the traditional practice of using native flora for medicinal purposes, which often included the use of leaves, roots, bark and flowers (Riley, 1994). Many plants were considered important in rongoā. The leaves of *P. colorata* (horopito) were used to treat toothache, skin irritations, and also as a painkiller (Brooker *et al*, 1987; Newton *et al*., 2000).

Pseudowintera colorata has been an integral part in rongoā medicine and to the Māori as an answer to fever, skin diseases, gonorrhea, stomach ache, toothache and also to wean infants (Brooker *et al*., 1987). The main biologically active chemical constituent of *P. colorata* has been identified as the sesquiterpene dialdehyde, polygodial (Fig. 1.2) (Mc Callion *et al.,* 1982), which has been shown to possess anti-fungal, and anti-bacterial properties. It is known that polygodial is a component of the "hot taste" in peppery spices common in traditional Japanese cuisine. Polygodial has been shown to exhibit fungicidal

activity against yeasts and filamentous fungi. A number of indigenous plant species of New Zealand have been studied for insect repellent properties and one study had found *P. colorata* to have insecticidal and antifeedant activity (Gerard and Ruf, 1991). The leaves of horopito are so hot to taste that sheep, cattle and deer usually avoid them and this hot taste has been associated with the insect antifeedant activity (Kubo and Ganjian, 1981). Other research suggests that increased red margin of the leaf is an indication of increased polygodial synthesis and a visual signal to reduce herbivory (Cooney *et al*., 2012). Polygodial was found to be a potent antifeedant against several lepidopteran species (Blaney *et al*., 1987; Schoonhoven and Fu-shun, 1989). Other research conducted by Gerard *et al*. (1993) proved that polygodial and another compound called 9-deoxymuzigadial were the main active compounds in *P. colorata* that exhibited antifeedant activity against insects *Tineola bisselliella* and the Australian carpet beetle (*Anthrenocerus australis*).

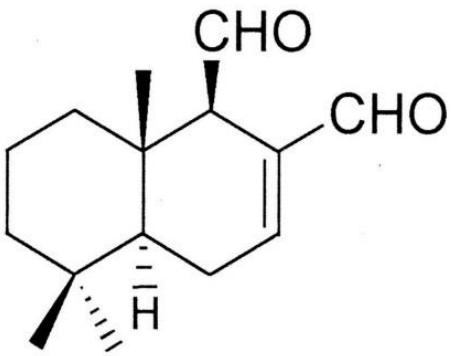


Figure 1.2: Polygodial (PubChem, NCBI, compound summary CID 72503)

1.6.2 Chemotype variations in *P. colorata*

Individual plants of *P. colorata* from four different locations were observed to have varying chemotypes with key differences in the level of polygodial (Wayman *et al*., 2010; Perry *et al*., 1996). Perry *et al*. (1996b) found that *P. colorata* plants from the South Island were of the mixed chemotype with both polygodial and 9-deoxymuzigadial, whereas *P. colorata* plants from the central North Island were predominantly of polygodial chemotype with little or no 9-deoxymuzigadial. Polygodial is commercially produced from the leaves of *P. colorata* and to date there are no published studies on the production of polygodial by any microorganism. The potential role of endophytes from *P. colorata* in the production of the compound has never been studied or demonstrated.

1.6.3 Commercial applications of polygodial from *P. colorata*

Kolorex® is the commercial product produced and marketed by New Zealand's Forest Herbs Research and is the first commercial compound using polygodial from horopito. It is used in treatment of *Candidiasis* and has been commercialized after Professor J.R.L. Walker and his team at New Zealand's University of Canterbury demonstrated that polygodial had strong anti-fungal activity against the yeast *Candida albicans*. Their study compared Amphotericin B to the polygodial extract from *P. colorata* and they observed larger zones of inhibition against *C. albicans* by the polygodial (Mc Callion *et al*., 1982). Pharmaceutical research has also indicated that usage of polygodial in patients suffering from chronic candidiasis decreased the instances of recurrence of candidiasis after a long-term usage with a higher cure rate in comparison to itraconazole (Fig. 1.3).

Kubo *et al*. (2005) demonstrated that polygodial had moderate antibacterial activity against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Salmonella typhi*. Previous research has also demonstrated strong antifungal activity against the yeast like fungi *Candida albicans, C. utilis, C. krusei, Cryptococcus neoformans, Saccharomyces cerevisiae* and also filamentous fungi including *Trichophyton mentogrophytes, T. ruburum* and *Penicillium marneffei* (Kubo and Lee, 1998).

Figure 1.3: Comparison of itraconazole and *P. colorata* (horopito) extract (polygodial) cure rates after 6 and 12 months respectively. From Journal of Biological Regulators & Homeostatic Agents. 2011 October-December; 25(4): 543-51.

1.7 Rationale

There have been no published studies to date about the endophytic communities of *P. colorata* and their functional traits. International research has indicated that the interactions between endophytes and their host plants can contribute to the (co-)production of bioactive molecules (Heinig *et al.,* 2013). Thus, there is the potential that endophytes may influence the production of polygodial in *P. colorata*. A key role for endophytes in New Zealand native plants is supported by the recent work of Wicaksono *et al.* (2016) who showed that endophytic bacteria recovered from *L. scoparium* had a key role in plant physiology and ecology. *In vitro* many of the endophytes from *L. scoparium* produced siderophores and showed antifungal activity against phytopathogenic fungi *Neofusicoccum luteum* and *Ilyonectria liriodendri*. Wicaksono *et al.* (2017a and b) also demonstrated that the endophytic bacteria isolated from *L. scoparium* were able to control the colonization of botrosphaeriaceous species when transferred to grapevine and reduced the disease intensity of *Pseudomonas syringiae* pv. *actinidiae* disease in kiwifruit.

1.8 Hypotheses of this study

The aim of this research is to describe the structure and function of the endophytic bacteria, Actinobacteria and fungi of *P. colorata* using culture dependent and culture independent techniques. The main hypotheses of this study were:

1) That the *P. colorata* endomicrobiome is affected by tissue type, maturity, and location.
2) That a core endomicrobiome is present within *P. colorata*.
3) That culturable endophytes of *P. colorata* produce bioactive compounds and possess functional properties that may be beneficial to the host.
4) That *P. colorata* harbours unique Actinobacteria and that Actinobacteria have a function *in planta*.
5) That culturable members of *P. colorata* endomicrobiome can influence the growth of *P. colorata* seedlings and have a potential role in plant chemistry.

Chapter 2

Structure and diversity of Actinobacterial, bacterial and fungal endophytes of *Pseudowintera colorata* (horopito)

2.1 Introduction

Pseudowintera colorata (horopito) is a medicinal plant that grows in the sub alpine regions of New Zealand and has been an integral part of traditional rongoā medicine. The leaves of *P. colorata* have been used in the treatment of fever, toothache, skin infections and gonorrhea (Brooker *et al.,* 1987). Like all land plants, it is likely to be inhabited by microbial endophytes and this community is collectively termed as the endomicrobiome. This population of microbes can confer unique characteristics to the host with a key influence on plant growth, development and disease resistance (Schutz, 2001). For example, the endophytic fungus *Piriformospora indica* colonizing roots of *Prosopis juliflora* (mesquite) and *Zizyphus mummularia* confers biotic and abiotic resistance to its plant hosts (Varma *et al.,* 1999; Waller *et al.,* 2005).

Although the use of medicinal plants as a source of biologically active compounds can be traced to ancient agricultural societies (Davis, 1995), the diversity of the endomicrobiome within the medicinal plants is still poorly understood (Nalini *et al.,* 2014). To date there is almost no information available on microbial associations with *P. colorata*.

The microbiome of *P. colorata* may function in enhancing the growth of the plant. Kumar *et al.* (2009) reported that the colonization of maize plants by the endophytic fungus *P. indica* led to increased growth and systemic resistance to the root pathogen *Fusarium verticilloides* via the enhancement of antioxidant defences within the host plant.

The first step to understanding the role of the microbiome in *P. colorata* is to identify the microbial communities that are found *in planta*. This is most effectively done using molecular tools, as many microorganisms are not culturable outside their host (Dinsdale *et al.,* 2008). For many years, the most common approach has been denaturing gradient gel electrophoresis (DGGE). In this process, PCR with taxa specific primers containing a GC clamp are arrayed on a denaturing gel. A study by Wicaksono *et al.* (2016) on another

native New Zealand medicinal plant *L. scoparium* (mānuka) used DGGE to study the structure and bioactivity of endophytic bacteria. Wicaksono *et al.* (2016) found that the plant tissue type affected structure and richness of the bacterial endophytic communities and that the bacterial communities stabilized and became uniform as the plants matured. Da Silva *et al.* (2013) used DGGE to study if the essential oils of *Lippia Sidoides* Cham. (pepper-rosmarin) affect its endophytic microbial communities and found that the total bacteria, Alphaproteobacteria, Betaproteobacteria and fungi were influenced both by the location inside the plant (leaves vs stems) and the presence of the main components of the *L. sidoides* essential oils in the leaves. Araújo *et al*. (2002) used DGGE to analyse the bacterial communities in symptomatic and asymptomatic citrus plants affected by citrus variegated chlorosis (CVC) caused by *Xylella fastidiosa.* The results showed a relationship between CVC symptoms and the frequency of isolation of the bacteria *Curtobacterium flaccumfaciens* suggesting a role for this organism in CVC disease resistance. Garcias-Bonet *et al*. (2012) used DGGE to analyse the bacterial communities in the marine angiosperm *Posidonia oceanica* and found that root communities significantly differed from rhizomes and leaves.

More recently, DGGE analysis has been displaced by new sequencing technologies such as Illumina MiSeq, which provides a deeper observation by detecting more species with greater accuracy compared to DGGE (Yu *et al*., 2015; Qin *et al*., 2016). Using Illumina MiSeq in *Aloe vera* plant, Akinsanya *et al*. (2015) found that *Proteobacteria, Firmicutes, Actinobacteria* and *Bacterioidetes* were the predominant genera and that roots of *A. vera* had the largest composition compared to other tissues. Using Illumina MiSeq platform, Wicaksono (2016) found that Gammaproteobacteria was the most abundant class in the tissues of *L. scoparium*.

Despite the number of international studies that detail the critical importance of the plant microbiome to the ecology and success of the plant there are almost no studies on the microorganisms associated with significant New Zealand native plants. These may be important from a restoration and conservation context as the endophytes may be unique and only associate with the native plants and play a pivotal role in the plant. This chapter presents the first study describing the endophytes of *P. colorata*.

The main objectives of this chapter were to:

1) Understand the diversity of the *P. colorata* endomicrobiome across multiple sites using denaturing gradient gel electrophoresis (DGGE) and identify members of the endomicrobiome that are affected by site and host tissue.
2) Analyze the diversity and composition of *P. colorata* endomicrobiome in mature plants from 10 sites across New Zealand using next generation sequencing (NGS) with Illumina MiSeq and identify a core endomicrobiome that is independent of site.

2.2 Materials and Methods

2.2.1 Sampling locations

Pseudowintera colorata samples were collected from 10 sites across New Zealand (6 South Island sites and 4 North Island sites) (Fig. 2.1) (Appendix A.1). The sites chosen for sampling were national parks and forest reserves maintained by the New Zealand Department of Conservation (DOC) to mitigate the possibility of external factors like urbanization affecting the plant and the endomicrobiome.

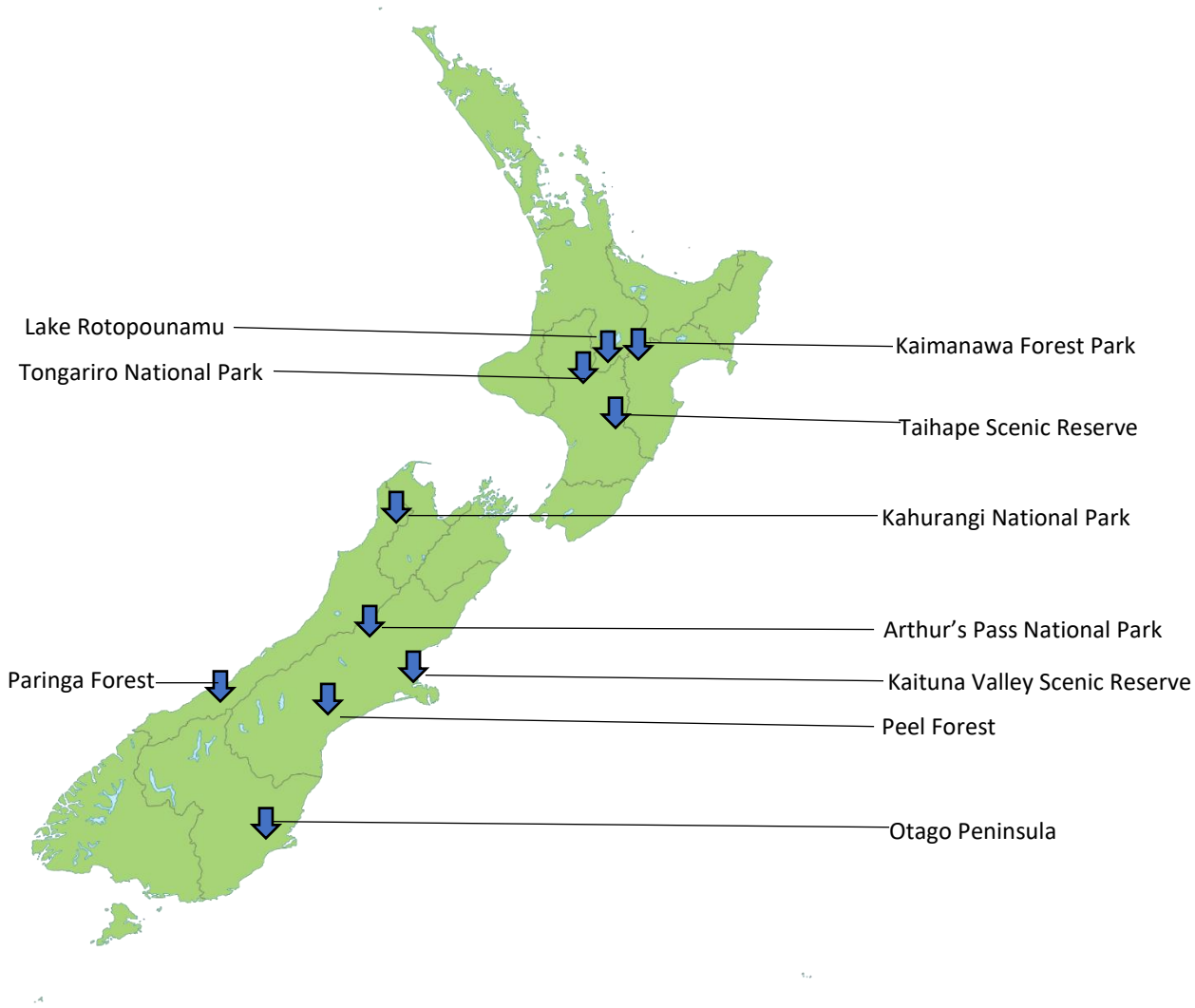


Figure 2.1: New Zealand map showing the sampling sites for *Pseudowintera colorata.*

2.2.2 Plant sampling

The leaves, stems and roots of *P. colorata* were sampled for the study. *Pseudowintera colorata* grows up to 10 m tall as a tree or 1 to 2.5 m as a shrub (Salmon, 1996). Since *P. colorata* is a very slow growing plant and the age of the sampled plants was unknown, the plants were classified as mature (>3 m), intermediate (<3 m >1 m), and young plants (≤1 m) based on their height. Wherever possible, at least three plants of each maturity were sampled per site in this study.

The leaf and stems tissues were cut using sterile secateurs from approximately 1 m above the ground. Whenever possible branches were sampled from opposite sides of the same plant. The selected leaves were fully open, mature, and free from any visible herbivory or

disease damage. Since mature *P. colorata* has a woody stem, lateral branches with green succulent growth were selected and cut using secateurs. For root samples, soil was excavated close to the plant and putative lateral roots belonging to *P. colorata* were traced back to the plant being sampled. The lateral root along with root hairs were collected for analysis. The tissues were collected into separate Ziploc bags, labelled and placed in an ice-bin. If the sampling times exceeded 24 h, the samples were stored at 4°C until processed.

2.2.3 Sample processing

All plant tissues were washed with tap water for approximately 15-30 s to remove soil and other debris and air-dried for 30-45 s on a clean paper towel. The tissues were surface sterilized with 96% ethanol for 10 s, followed by immersion in 2.5% freshly prepared sodium hypochlorite for 3 min and three consecutive washes with sterile distilled water for 1 min each. The surface sterilized tissues were used for isolation of culturable endophytes and will be described in Chapter 3. Portions of each sterilised plant tissue were set aside and preserved in 50 mL tubes containing 20% sterile glycerol and stored at -80°C for extraction of DNA for DGGE and NGS.

2.2.4 DNA extraction

Surface sterilized *P. colorata* tissues (leaves, stem and root) were treated with propidium monoazide (PMA) to exclude any residual surface DNA from amplification by PCR (Nocker *et al*., 2007). Tissue samples preserved in 20% glycerol and stored at -80°C were thawed before use.

The leaf samples were cut transversely from one side to the other to allow maximum surface coverage. The slivers were then added to a separate tube containing PCR grade sterile water. The tissues were then treated with 1.25 µL PMA to eliminate any surface DNA. The tubes were mixed well by inverting and incubated in the dark for 5 min with occasional mixing.

The tubes were laid on a reflective tray that was placed on an ice pack and exposed to a halogen bulb for 10 min to activate the PMA. The tubes were placed 15- 20 cm away from the light source to avoid overheating the tissue samples.

Since *P. colorata* is rich in polyphenolic compounds DNA was extracted using a modified CTAB method to provide DNA of sufficient quality for both DGGE and Illumina MiSeq (Allen

et al., 2006). In this process, the samples were snap frozen in liquid nitrogen and then ground to a fine powder in a pre-chilled mortar and pestle. Chilling avoided the release of polyphenolic compounds from the plant material. Approximately 200 mg of the freshly ground sample was added to chilled tubes using a chilled spatula and placed into liquid nitrogen. Tubes were removed from the liquid nitrogen and 1.2 mL freshly prepared CTAB buffer (Appendix A.2) containing 2% polyvinylpyrrolidone (PVP 40) and 2% beta mercaptoethanol (βME) added and the tubes inverted at least 50 times to mix the buffer and sample. The tubes were then incubated at 65°C for 30 min and mixed by inverting every 5-10 min. After incubation, the tubes were centrifuged at 13500 X *g* for 10 min and the supernatant carefully pipetted into a separate tube containing 800 µL of chloroform: isoamylalcohol (24:1). The tubes were gently mixed by inverting several times and incubated at room temperature for 20 min with occasional mixing. The tubes were then centrifuged at 13500 X *g* for 10 min to separate the phases. The aqueous layer was pipetted into a new tube containing 800 µL ice-cold isopropanol. The tubes were incubated at room temperature for 10 min and then centrifuged at 13500 X *g* for 10 min. The isopropanol was carefully decanted without disturbing the pellet. The pellet was then washed in 500 µL of 70% ice-cold ethanol and centrifuged at 13500 X *g* for 10 min. The ethanol was decanted and the tubes were inverted to dry on a sterile surface for 1 h. The pellet was resuspended in 30 µL of PCR grade water and left to hydrate at 4°C. The quality and yield of DNA was determined by electrophoresis of the samples in 1.5% agarose and spectrophotometry using Qubit DNA ds BR Assay system (Thermo Fisher Scientific Inc., New Zealand) by following the manufacturer's instructions.

To concentrate the DNA extracted from stem and root tissues, ethanol precipitation with sodium acetate was used. To each tube 1/10 volume of 3 mM sodium acetate and 2-2.5 volumes of 99% ice-cold ethanol (-20°C) was added and incubated at -20°C for 1 h. The tubes were then centrifuged at 13500 X *g* for 12-15 min. The supernatant was decanted and the pellet was washed in 1 mL 70% ice-cold ethanol and centrifuged at 13500 X *g* for 2 min. The supernatant was carefully decanted and the tubes air-dried for 1 h by inverting on a sterile surface. The pellet was resuspended in 20 µL of PCR grade water and left to hydrate overnight at 4°C. The DNA was used for Illumina MiSeq analysis and for PCR with group specific primers and the products were separated using DGGE.

2.2.5 Analysing the endomicrobiome structure using DGGE

DNA was amplified using group specific primers for Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and total fungi (Table 2.1). The PCR products were separated by DGGE, the presence and absence of bands recorded.

Table 2.1 Sequence details of the group-specific 16S rRNA and ITS gene primers used for PCR.

Group	Primer	Sequence (‘5 - 3’)	References
α-Proteobacteria	F203α	CCG CAT ACG CCC TAC GGG GGA AAG ATT TAT	Nübel *et al*. (1996), Gomes et al. (2001)
	L1401	CGG TGT GTA CAA GAC CC	
	341F-GC	GC–CCT ACG GGA GGC AGC AG	
	518R	ATT ACC GCG GCT GG	
Actinobacteria	F243	GGA TGA GCC CGC GGC CTA	Heuer et al. (1997), Nimnoi et al. (2010)
	1494R	TAC GGC TAC CTT GTT ACG AC	
	341F-GC	GC–CCT ACG GGA GGC AGC AG	
	R534	ATT ACC GCG GCT GG	
β-Proteobacteria	Beta359F	GGG GAA TTT TGG ACA ATG GG	Ashelford *et al.* (2002), Mühling *et al*. (2008)
	Beta682R	GGG GAA TTT TGG ACA ATG GG	
	518F-GC	GC–CCA GCA GCC GCG GTA AT	
γ-Proteobacteria	Gamma395F	CMA TGC CGC GTG TGT GAA	Lee *et al.* (1993), Mühling et al. (2008)
	Gamma871R	ACT CCC CAG GCG GTC DAC TTA	
	518F-GC	GC–CCA GCA GCC GCG GTA AT	
	785R	CTA CCA GGG TAT CTA ATC C	
Total Fungi	AU2	TTT CGA TGG TAG GAT AGD GG	VandenKoornhuyse *et al.* (2002), Vainio and Hantula (2000)
	AU4	RTC TCA CTA AGC CCA TTC	
	FF390	CGA TAA CGA ACG AGA CCT	
	FR1-GC	GC–AIC CAT TCA ATC GGT AIT	

2.2.5.1 Actinobacterial communities

The protocol from Nimnoi *et al.* (2010) was slightly modified for this study in the usage of primer 1494R (Stark *et al.,* 2007) instead of R1378. To amplify the 16S rRNA from only actinobacteria, the specific primer F243 was used in the first PCR along with the universal bacterial reverse primer 1494R. The PCR products from the primer pair F243-R1494 were then used as templates for a second PCR with the primer pair F341-GC and R534. This primer pair was selected because it amplifies the variable region (V3) within the 16S rRNA. The primary PCR were performed in 25 μL reaction volumes, containing 1 μL of template DNA, 2.5 μL buffer, 200 μM dNTPs, 1 U *Taq* DNA polymerase, 1 μL of each primer (10 μM) and 18.75 μL water. Positive and negative controls were run for each PCR. The PCR amplification conditions were as follows: initial denaturation was performed at 95°C for 5 min, followed by denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and chain elongation at 72°C for 2 min. These three steps were repeated for 35 cycles. Final elongation was performed at 72°C for 10 min and the reactions were cooled to 4°C.

The secondary PCR were performed in 25 μL reaction volumes as previously described except the DNA was replaced by 1 μL of product from the first PCR. The PCR amplification conditions were as follows: initial denaturation was performed at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 53°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min.

The amplified products were separated on 1% (w/v) agarose gels in 1 x TAE (Appendix A.3) at 100 V for 1 h, stained in ethidium bromide (EtBr; Appendix A.4) and visualized under UV light.

2.2.5.2 Alphaproteobacteria

The protocol for the amplification of Alphaproteobacteria was also slightly modified with the initial denaturation step of the first PCR being performed at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min, with final extension at 72°C for 10 min. The primers used for the first PCR were F203α and L1401 and the PCR mix was the same as described in Section 2.2.5.1. The secondary PCR was performed using the primer pair 341F-GC and 518R using the PCR product from the first PCR as template. The conditions for the secondary PCR were similar

to the first cycle except for 35 cycles of denaturation, annealing at 63°C for 1 min, extension at 72°C for 1 min, with final extension at 72°C for 5 min.

2.2.5.3 Betaproteobacteria and Gammaproteobacteria

The primer pairs Beta359F - Beta682R and 518F GC - Beta682R, Gamma395F - Gamma871R and 518F-GC -785R were used in the first and second PCR cycles for amplifying Beta and Gammaproteobacteria communities, respectively. PCRs (both primary and secondary) for Betaproteobacteria, Gammaproteobacteria used the same cycle protocol and PCR mix except for the variations in the annealing temperature. Following an initial denaturation step of 4 min at 96°C, 30 PCR cycles were performed at 96°C for 1 min, annealing temperature for 1 min, 74°C for 1 min, followed by a final extension step at 74°C for 10 min. Where annealing temperature was 63°C and 60°C for Betaproteobacteria, 54°C and 56°C for Gammaproteobacteria primary and secondary PCRs respectively.

2.2.5.4 Total Fungi

The PCR for analysing the fungal communities was performed using the primer pair AU2 and AU4. The PCR product from the primer pair AU2-AU4 was used as template for a second PCR with primer pair FF390 and FR1-GC. PCR of the primer pair AU2-AU4 were performed in 25 μL reaction volumes containing reagents as previously described. Positive and negative controls were run for each PCR. The PCR amplification conditions were as follows: initial denaturation was performed at 95°C for 3 min, followed by denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and chain elongation at 72°C for 1 min. These three steps were repeated for 35 cycles. Final elongation was performed at 72°C for 7 min.

PCR for the primer pair FF390 and FR1-GC were performed in 25 μL reaction volumes as previously described with 1 μL of PCR product from the first PCR as template. The PCR amplification conditions were as follows: initial denaturation was performed at 95°C for 2 min; followed by 8 cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min. This was followed by 27 cycles of denaturation at 95°C for 30 s, primer annealing at 47°C for 30 s, and extension at 72°C for 1 min, and a final extension step at 72°C for 7.5 min.

The amplified products from all the PCR's were separated on 1% (g/ml) agarose gels, stained with ethidium bromide (EtBr) and visualized under UV light.

2.2.6 DGGE

DGGE was performed using a Cipher DGGE Electrophoresis system (CBS Scientific). Gel bond (SERVA, Germany) was placed on clean DGGE glass plates, while ensuring that the bond adhered to the glass plate fully and there were no bubbles between the bond and the glass before pouring the gradient gel. A linear gradient gel containing 10 mL low and 10 mL high gradient of 8% (w/v) polyacrylamide gel (acrylamide/bis solution, 37.5:1) i.e., 40 to 60% for Alphaproteobacteria (Da Silva *et al.,* 2013) and Gammaproteobacteria, 40 to 55% for Betaproteobacteria (Mühling *et al*., 2008), 35 to 50% for Actinobacteria (Nimnoi *et al.,* 2010; Nimnoi *et al.,* 2011), 25 to 55% for total fungi (Vainio and Hantula 2000); 100% denaturant was defined as 7 M urea (Sigma-Aldrich, Sigma-Aldrich Co. LLC) and 40% (v/v) formamide (Sigma-Aldrich, Sigma-Aldrich Co. LLC), were poured into DGGE glass plates. Each gradient solution contained 0.0012% (v/v) tetramethylethylenediamine (TEMED) and 0.07% (w/v) ammonium persulfate (APS) (Biorad, New Zealand) as catalysts for polymerization of acrylamide and bis-acrylamide. After 30 min of gradient gel polymerization, 5 ml of stacking gel solution containing 0% denaturant polyacrylamide gel (acrylamide/bis solution, 37.5:1), 0.002% TEMED and 0.1% APS were poured onto the gradient gel with the DGGE gel comb placed between the glass plates.

Eight µL of PCR product with 8 µL of loading dye (0.5% bromophenol blue, 0.5% xylene cyanol and 70% glycerol in ddH_20) were loaded onto the gradient gel. The gels were run in 0.5 × TAE buffer for 16 h at 65 V for Alpha, 18 h at 60 V for Beta and Gammaproteobacteria, 6 h at 130 V for Actinobacteria and 15 h at 90 V for total fungi. A sample from the collection (leaf sample from Tongariro) was run in the corner lanes as a reference standard. After the gels were run, the DGGE glass plates were removed and the gel bonds with the gels were placed on a clean gel tray and 200-250 mL of fixative solution (40% ethanol, 2% acetic acid in water) was added and the gel was rocked for 3 min. The gels were then stained using 200-250 mL of silver stain solution (0.1% (w/v) silver nitrate) for 10 min. The excess silver stain from the gels was removed and the gels were rinsed with Millipore water for 2 min. The gels were then developed with 200-250 mL of developer solution (3% (w/v) sodium hydroxide and 0.01% (v/v) formaldehyde solution in Millipore water) for 45 min. The gels

were then shaken with 200-250 mL fixative solution for 5 min and rinsed using Millipore water for 2 min. Finally, the gels were soaked in 200-250 mL of Cairn's preservation solution (25% ethanol, 10% glycerol in water) and covered with GelAir cellophane membrane (BioRad) and placed for drying in an oven at 65^{o}C for 24-48 h. The gel was then scanned and the converted file (.jpeg) was used for further analysis.

The microbial communities were analysed using Phoretix 1D Pro Gel Analysis (Totallab, UK), which generated a matrix based on the presence/absence of bands in each sample. The resultant band matrix was analysed by Primer version 7 (Primer-E Ltd, Plymouth Marine Laboratory, UK) multivariate software package. Using Jaccard coefficient, a resemblance matrix based on similarity was generated (Clarke and Warwick 1994). The nonmetric multidimensional scaling (nMDS) ordination, main and pair-wise PERMANOVA tests were performed to test the statistical difference between endophytic bacterial and fungal communities among samples. Taxon richness was calculated based on the number of bands per lane, where each band was considered as one bacterial/fungal taxon. A general linear model (GLM) was used to calculate the richness of bacterial and fungal communities, followed by Fisher's ad-hoc analysis at $P<0.05$ using Minitab 17 (Lead Technologies, Australia) as described (Wicaksono et al. 2016).

2.2.7 Illumina MiSeq metabarcoding of microbial endophytes of *P. colorata*

For the Illumina MiSeq sequencing, the final composite DNA samples were prepared by measuring and pooling the DNA extracted from the same tissue type of multiple individual plants collected at the same site (Appendix A.5). This was done to reduce the processing costs involved for the NGS run. In total, 31 *P. colorata* tissue samples (leaves, stems and roots) representing 10 sites across New Zealand were obtained by pooling the DNA from 87 individual *P. colorata* tissues (leaves, stems and roots). The minimum concentration of DNA for Illumina MiSeq as communicated by New Zealand Genomics Ltd (NZGL; Patrick Biggs, pers comm.) was 10 ng/µL. The V3-V4 hypervariable region of the 16S rRNA gene and ITS2 region of the ITS gene region of *P. colorata* endophytic bacterial and fungal communities, respectively were amplified using the primers 341F (5'–CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'), and fITS7 (5'-GTGARTCATCGAATCTTTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') respectively, as recommended by NZGL (Patrick Biggs, pers comm.) (Appendix A.6 and A7). The PCR

amplification protocol was adapted from Klindworth *et al.* (2012). The primers included the Illumina cell flow adaptors and unique barcodes for identifying the samples within the amplicon libraries. The PCR were performed in a total volume of 25 μL and contained 12.5 μL of 2X KAPA HiFi HotStart ReadyMix (Kapa Biosystem, South Africa), 5 μL each of the forward and reverse primer stock (1 mM) and 2.5 μL of genomic DNA at a concentration of 5 ng/μL. The PCR were carried out in an Applied Biosystems Proflex PCR system, set up in triplicates and the resulting products pooled per sample. The samples were purified using Agencourt AMPure XP- PCR purification kit (Beckman Coulter, New Zealand). The resulting libraries were quantified using the Qubit DNA ds BR assay system as per the manufacturer's protocol (Thermo Fisher Scientific, USA). Amplicon libraries were prepared from *P. colorata* leaves, stems and root samples from each of the 10 sites across New Zealand (Fig. 2.1). The amplicon libraries were sequenced by NZGL using the Illumina MiSeq v2 platform (250 bp paired end).

The raw data from the sequencing was mapped using Bowtie2 and against bacteriophage PhiX genome, which was used as a control in the Illumina sequencing runs. Sequences that matched the PhiX sequences were removed from the Sequence Alignment Map (SAM) file and the resultant fastq files were reconstructed using SamToFastq.jar program in the Picard suite (http://broadinstitute.github.io/picard/). The Illumina adaptors were removed using the fastq-mcf program (version 1.1.2-621) from ea-utils tool suite (http://code.google.com/p/eautils/). Paired end reads were joined and the sequences were filtered based on their quality and length (Phred score $\geq$ 15 and 400-450 bp). Any chimeric sequences were removed by checking the sequences against RDP Gold reference database (http://drive5.com/uchime/rdp_gold.fa) using a USEARCH v8.1 script (Edgar *et al*., 2011) in QIIME 1.8.0 open source software package (http://qiime.org). The RDP gold database is a chimera-free reference database and contains 10,049 reference sequences. The reads were clustered as operational taxonomic units (OTUs) using the UCLUST algorithm, keeping the default parameters, which was 97% similarity based on Edgar (2010). The representative sequences were then assigned taxonomy by the RDP naïve Bayesian rRNA classifier using 80% threshold (Wang *et al*., 2007) using the Greengenes reference database (version gg_13_8_99) (DeSantis *et al*., 2006). Reads that were assigned as either chloroplast or mitochondria were excluded from further analysis. The sequence

number for each of the samples was normalized to the lowest number of read counts selected from a random subset of sequences generated using a script provided in the QIIME 1.8.0 software package (Appendix A.8). To calculate the alpha diversity, the bacterial richness was estimated using the number of observed OTUs and the bacterial diversity was determined using the Simpson index in the QIIME 1.8.0 software. The taxonomy bar charts were generated and visualized using PHINCH open source framework (Bik and Pitch interactive 2014). Beta diversity was assessed by performing principal coordinate analysis (PCoA) based on calculating the weighted normalized UniFrac distance matrix (Lozupone and Knight, 2005) using Primer 7 software.

To identify if there was a core endomicrobiome present in *P. colorata*, the script compute_core_microbiome on QIIME 1.8.0 was used to filter OTUs that were found in $\geq$ 75% of plant tissues.

2.2.8 Functional prediction of *P. colorata* endomicrobiome using phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt)

To predict the possible functions of bacterial endophytes in *P. colorata* an open source tool called PICRUSt (http://picrust.github.com) (Langille *et al*., 2013) was used. PICRUSt uses 16S rRNA abundances to predict the gene families. Before using the function prediction analysis in PICRUSt, the abundances of different 16S rRNA genes were normalized based on the known gene copy number for that OTU.

2.3 Results

2.3.1 Analysis of the endomicrobiome structure using DGGE

2.3.1.1 Actinobacteria

Plant tissue, location (n=10) and the interactions between location and plant tissue influenced the actinobacterial communities (PERMANOVA $P \leq 0.05$) (Table 2.2). Roots and stems were more tightly clustered, whereas, the leaves were more scattered (Fig. 2.2). The number of bands were higher in the stem samples compared to other tissues (LSD, $P \leq 0.05$) (Table 2.3). Actinobacterial taxa were richer in stems (n=18) and leaves (n=16) compared to roots (n=13) (LSD, $P \leq 0.05$) (Table 2.3). Plant location influenced the richness in stems (PERMANOVA $P \leq 0.05$) and leaves (PERMANOVA $P \leq 0.005$), but not roots (PERMANOVA $P=0.255$).

Table 2.2 Effect of plant location and plant tissue on the similarity of microbial communities of *Pseudowintera colorata*

Treatment	†Microbial communities similarity				
	Actinobacteria	α proteobacteria	β proteobacteria	γ proteobacteria	Total Fungi
Location	0.007*	0.323	0.149	0.312	0.081
Plant tissue	0.001**	0.001**	0.001**	0.001**	0.001**
Location vs plant tissue	0.002**	0.021*	0.001**	0.100	0.002**

†Asterisk denotes levels of statistical significance of microbial communities similarity based on PERMANOVA. *significantly different ($P \leq 0.05$), **high significant difference ($P \leq 0.005$)

Table 2.3 Effect of plant location and plant tissue on the microbial richness of *Pseudowintera colorata*

Treatment	Microbial Richness				
	Actinobacteria	α proteobacteria	β proteobacteria	γ proteobacteria	Total Fungi
Location	0.177	0.036*	0.756	0.204	0.095
Plant tissue	0.045*	<0.001**	<0.001**	<0.001**	<0.001**
Location vs Plant tissue	0.298	0.253	0.057	0.164	<0.001**

†Asterisk denotes levels of statistical significance of microbial communities richness based on GLM. *significantly different ($P \leq 0.05$), **highly significant difference ($P \leq 0.005$)

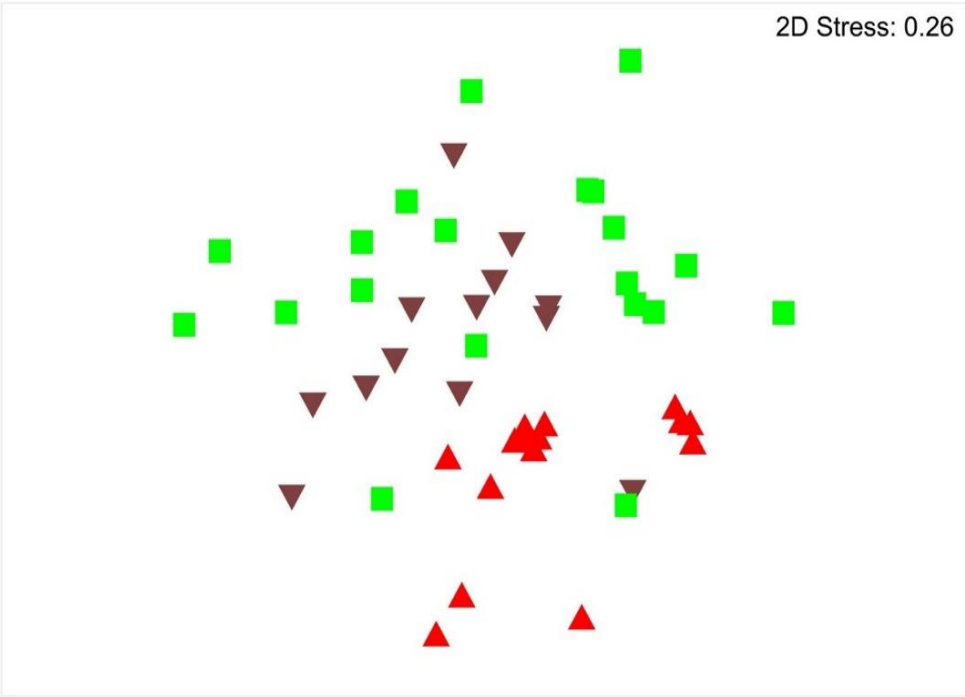


Figure 2.2: Nonmetric multidimensional scaling (MDS) plot showing actinobacterial communities from different plant tissues of *Pseudowintera colorata*. Leaf (■), Stem (▲), Root (▼).

2.3.1.2 Alphaproteobacteria

Plant tissues and interaction with location influenced Alphaproteobacteria communities (PERMANOVA, $P \leq 0.05$ (Table 2.2). Leaves, stems and roots formed discrete clusters (Fig. 2.3). Leaves (n=18) and roots (n=15) had higher Alphaproteobacteria richness compared to stems (n=14) (LSD $P \leq 0.005$) (Table 2.3).

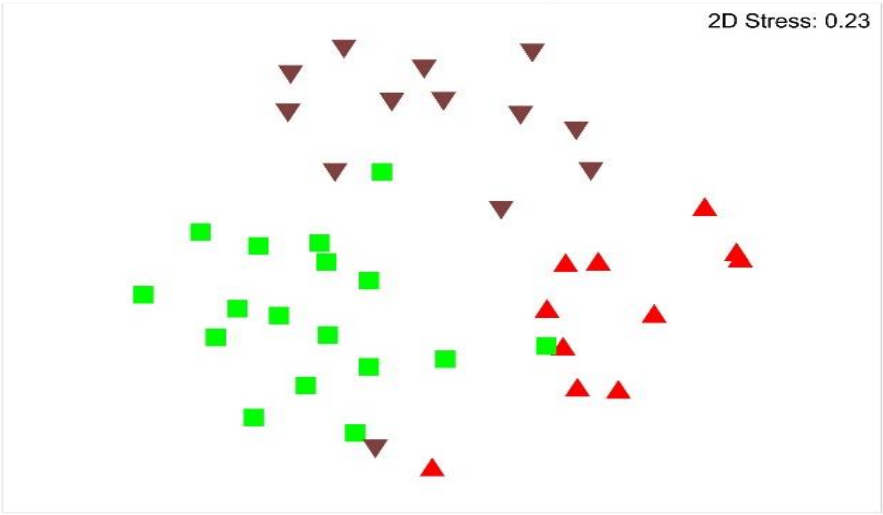


Figure 2.3: Nonmetric multidimensional scaling (MDS) plot showing Alphaproteobacteria communities from different plant tissues of *Pseudowintera colorata*. Leaf (■), Stem (▲), Root (▼).

2.3.1.3 Betaproteobacteria

Plant tissue and interaction with location influenced Betaproteobacteria communities (PERMANOVA, $P \leq 0.005$) (Table 2.2). The stems clustered together, whereas, the leaves and roots were more scattered (Fig. 2.4). The richness of Betaproteobacteria was higher in stems (n=18) compared to roots (n=12) and leaves (n=10) (LSD, $P \leq 0.005$) (Table 2.3).

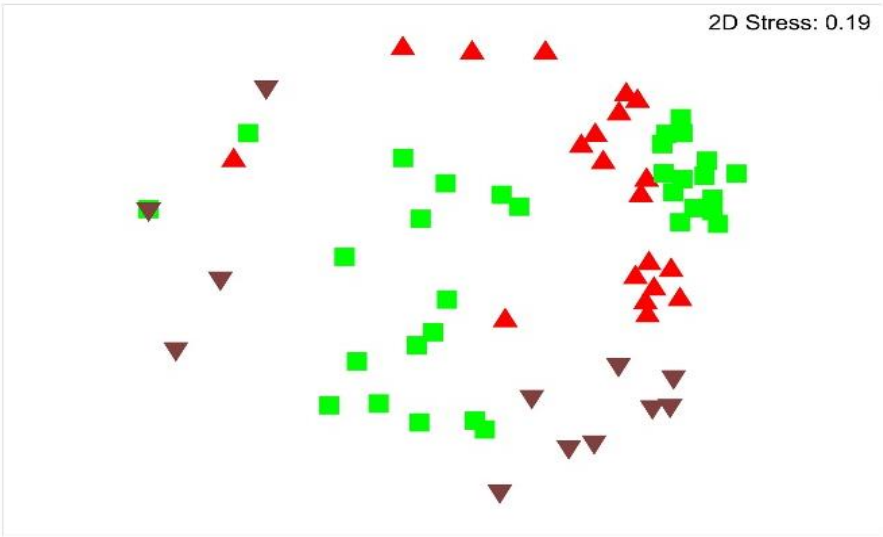


Figure 2.4: Nonmetric multidimensional scaling (MDS) plot showing Betaproteobacteria communities from different plant tissues of *Pseudowintera colorata*. Leaf (■), Stem (▲), Root (▼).

2.3.1.4 Gammaproteobacteria

Plant tissue influenced the Gammaproteobacteria communities (PERMANOVA, $P \leq 0.005$) (Table 2.2). Stems, roots and leaves formed separate clusters, with the leaves being more diverse (PERMANOVA, $P \leq 0.005$) (Fig. 2.5). The richness of Gammaproteobacteria was the greater in the roots (n=19) compared to stems (n=12) and leaves (n=10) (LSD, $P \leq 0.005$) (Table 2.3).

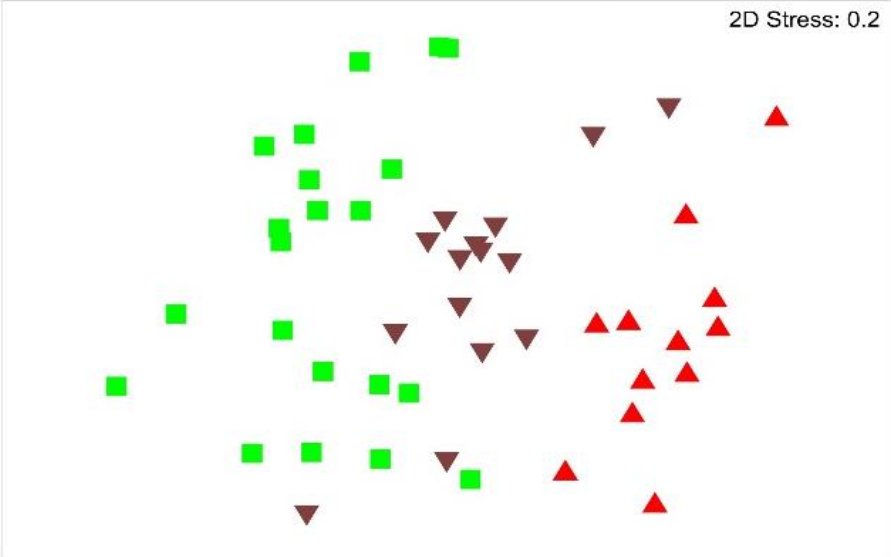


Figure 2.5: Nonmetric multidimensional scaling (MDS) plot showing Gammaproteobacteria communities from different plant tissues of *Pseudowintera colorata*. Leaf (■), Stem (▲), Root (▼).

2.3.1.5 Total Fungi

Plant tissue and interaction with location influenced the total fungal communities (PERMANOVA, $P \leq 0.005$) (Table 2.2). The stems clustered together, while the leaves and roots were more scattered (Fig 2.6). The stems (n=21) were richer in fungal taxa compared to the roots (n=16) and leaves (n=7) (LSD, $P \leq 0.005$) (Table 2.3). Location did not influence the richness of the fungal communities (Table 2.3).

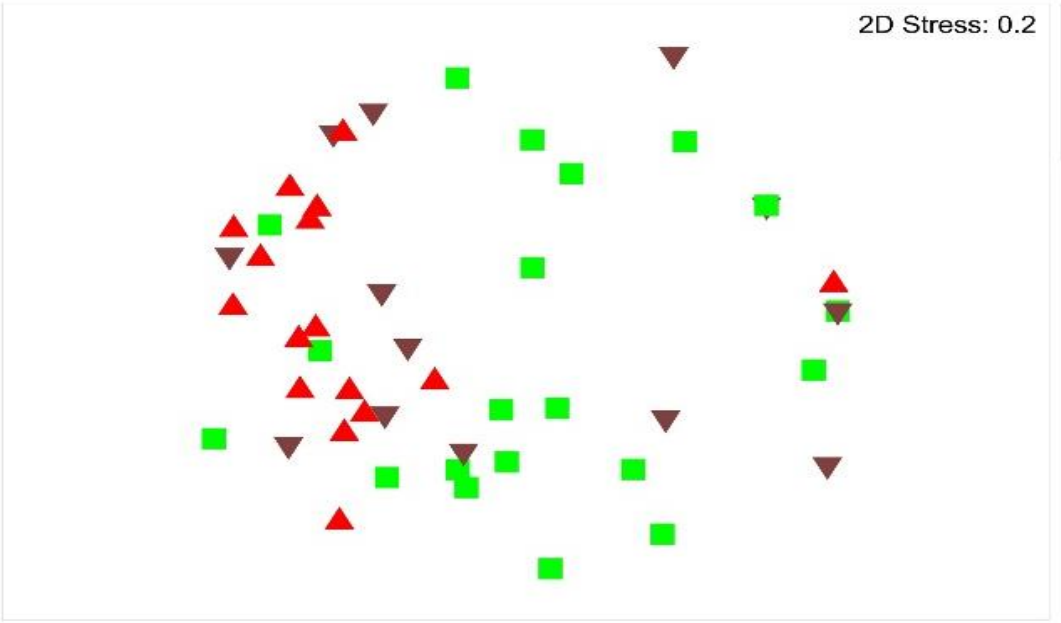


Figure 2.6: Nonmetric multidimensional scaling (MDS) plot showing fungal communities from different plant tissues of *Pseudowintera colorata*. Leaf (■), Stem (▲), Root (▼).

2.3.2 Effect of plant maturity on the endophyte community structure and richness in *P. colorata*

Since the sites chosen for this study were restricted to DOC sites only, collection of immature plants was not always possible. Thus, for analysing the influence of plant maturity on the community structure of endophytes in *P. colorata*, a subset of three sites were selected where plants of different maturities were present. These were Kaituna Valley Forest Park, Paringa Forest and Peel Forest. The plants collected at these sites were classified based on their height as mature plants (>3 m) and immature plants (≤1 m).

2.3.2.1 Actinobacteria

Plant tissue, location, maturity and the interaction between these factors influenced actinobacterial communities (PERMANOVA $P \leq 0.05$) (Table 2.4). The leaves and stems of immature plants and mature plants did not cluster (PERMANOVA, $P=0.246$ and $P=0.044$ respectively), while only the roots of immature plants formed discrete clusters (PERMANOVA, and $P=0.001$) (Fig. 2.7). Actinobacterial taxa was richer in stems and leaves compared to roots (LSD, $P \leq 0.005$) (Table 2.5).

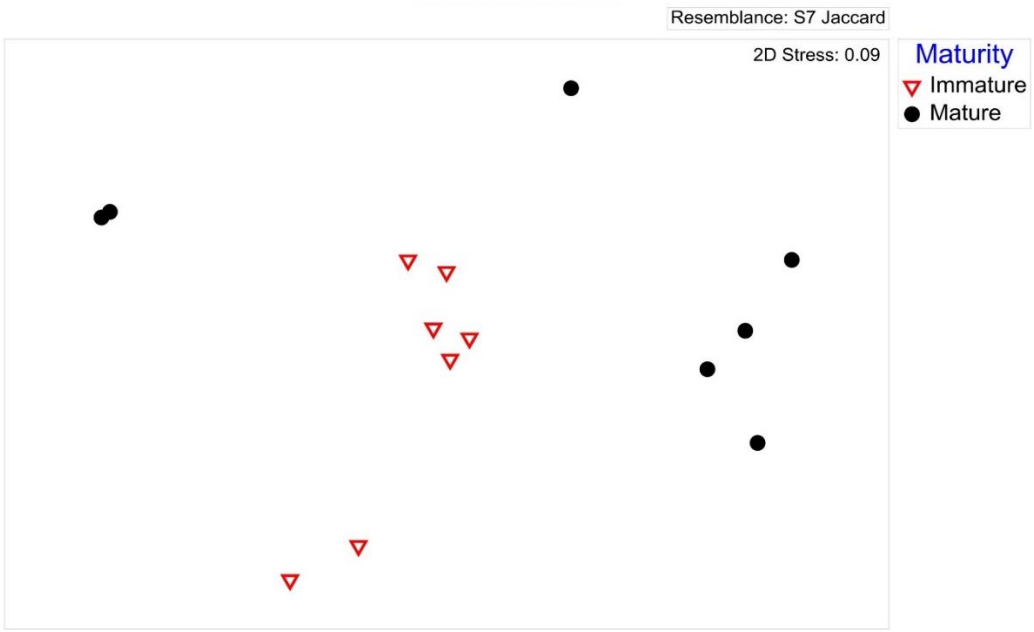


Figure 2.7: Nonmetric multidimensional scaling (MDS) plot showing actinobacteria communities from mature and immature plants roots of *Pseudowintera colorata*. Mature plant●; Immature plant▽.

Table 2.4 Effect of plant location (n=3), plant tissue and plant maturity on the similarity of microbial communities of *Pseudowintera colorata*

Treatment	Actinobacteria	α proteobacteria	β proteobacteria	γ proteobacteria	Total Fungi
Location	0.001**	0.001**	0.001**	0.019*	0.002**
Plant tissue	0.001**	0.001**	0.001**	0.001**	0.001**
Maturity	0.010*	0.532	0.001**	0.001**	0.002**
Location vs plant tissue	0.001**	0.001**	0.001**	0.001**	0.001**
Location vs Maturity	0.010*	0.226	0.005**	0.030*	0.164
Plant tissue vs maturity	0.001**	0.098	0.001**	0.001**	0.001**
Plant tissue vs location vs maturity	0.001**	0.001**	0.001**	0.001**	0.001**

Table 2.5 Effect of plant location (n=3), plant tissue and plant maturity of *Pseudowintera colorata* on the microbial richness

Treatment	Actinobacteria	α proteobacteria	β proteobacteria	γ proteobacteria	Total Fungi
Location	0.130	0.022*	0.017*	0.028*	0.121
Plant tissue	0.013*	<0.001**	0.009**	<0.001**	0.015*
Maturity	0.096	0.2	0.250	0.45	0.785
Location vs Plant tissue	<0.001**	0.001**	0.025*	<0.001**	<0.001**
Location vs maturity	0.178	0.08	0.196	0.252	0.448
Plant tissue vs maturity	0.920	0.112	<0.001**	0.247	0.393
Plant tissue vs location vs maturity	0.029*	<0.001**	<0.001**	0.125	0.060

†Asterisk denotes levels of statistical significance of microbial communities richness based on GLM. *significantly different ($P \leq 0.05$), **highly significant difference ($P \leq 0.005$).

2.3.2.2 Alphaproteobacteria

Location, plant tissue and the interaction with maturity influenced Alphaproteobacteria communities (PERMANOVA, $P \leq 0.005$) (Table 2.4). In contrast to actinobacteria there was no clustering according to maturity for any tissue (PERMANOVA, $P=0.532$) (Fig. 2.8). Alphaproteobacteria richness was higher in leaves (n=18) compared to roots (n=15) and stems (n=14). (LSD, $P \leq 0.05$) (Table 2.5).

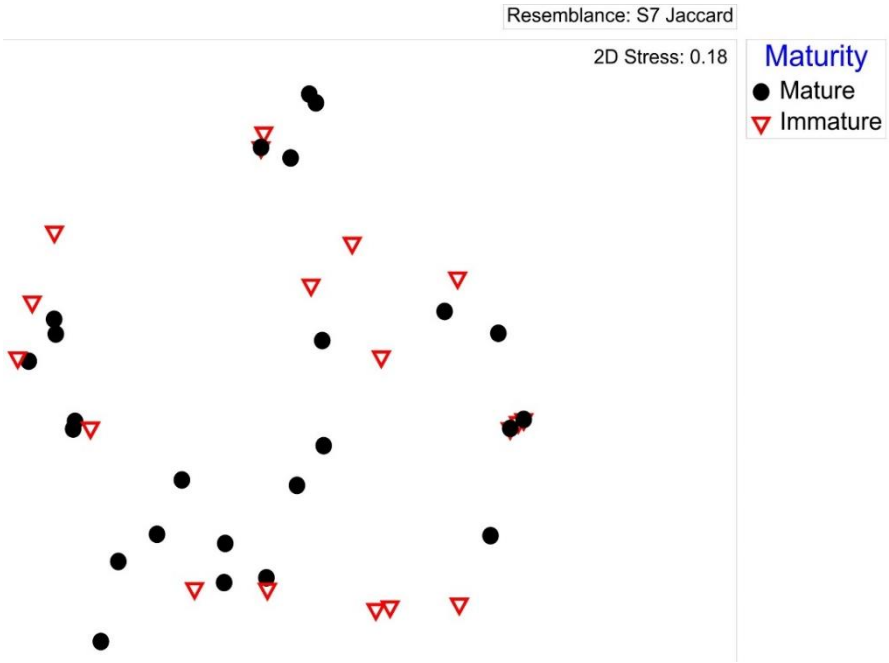


Figure 2.8: Nonmetric multidimensional scaling (MDS) plot showing Alphaproteobacteria communities from all tissues of mature and immature plants. Mature plant●; Immature plant▽.

2.3.2.3 Betaproteobacteria

All factors and their interactions influenced Betaproteobacteria communities (PERMANOVA, $P \leq 0.005$) (Table 2.4). The combined tissue data showed that immature plants were more diverse, while the mature plants clustered together (PERMANOVA, $P \leq 0.005$). Mature leaves were more diverse than the immature leaves (PERMANOVA, $P \leq 0.005$) (Fig. 2.9). The richness of Betaproteobacteria was higher in roots (n=18), compared to stems (n=12) and leaves (n=10) (LSD, $P \leq 0.05$) (Table 2.5).

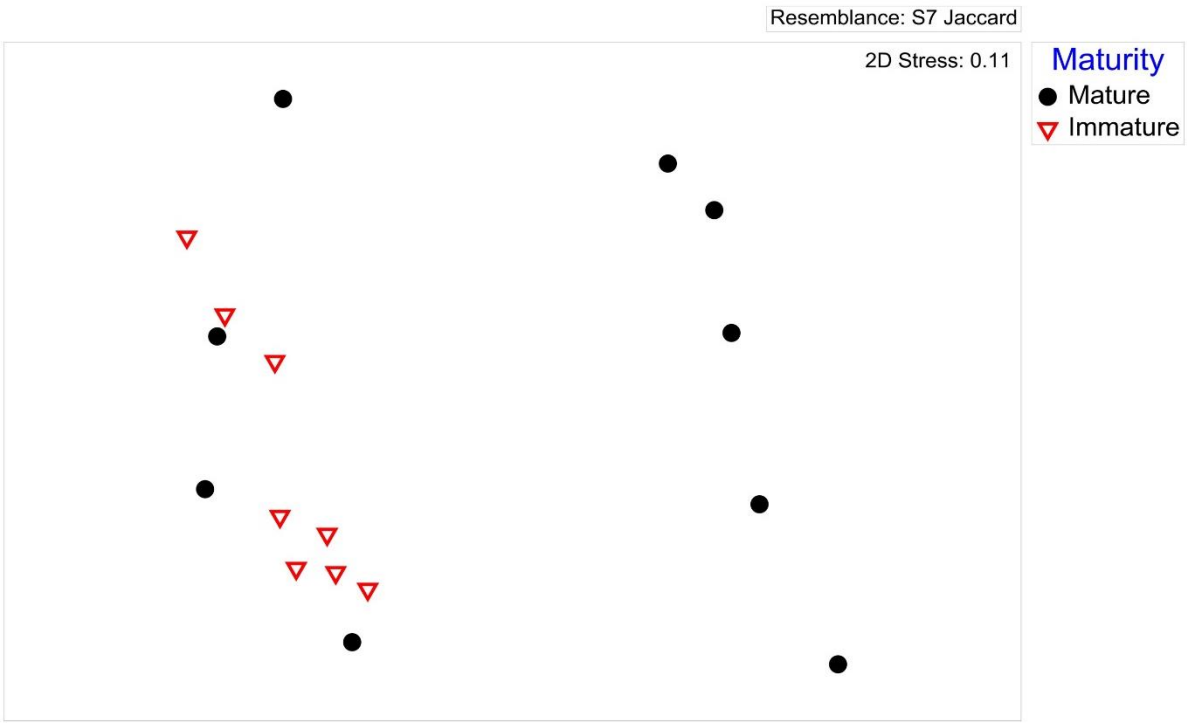


Figure 2.9: Nonmetric multidimensional scaling (MDS) plot showing Betaproteobacteria communities from mature and immature plants leaves of *Pseudowintera colorata*. Mature plant●; Immature plant▽.

2.3.2.4 Gammaproteobacteria

All factors and their interactions influenced the Gammaproteobacteria communities (PERMANOVA, $P \leq 0.05$) (Table 2.4). When data for all tissues was combined, immature plants showed no specific clustering, while the mature plants formed clusters (PERMANOVA, $P=0.001$). The leaves of mature and immature plants clustered separately (PERMANOVA, $P=0.001$) (Fig. 2.10). The richness of Gammaproteobacteria was greater in the roots (n=19) compared to leaves (n=10) and stems (n=12), with roots> leaves> stems (LSD, $P \leq 0.005$) (Table 2.5).

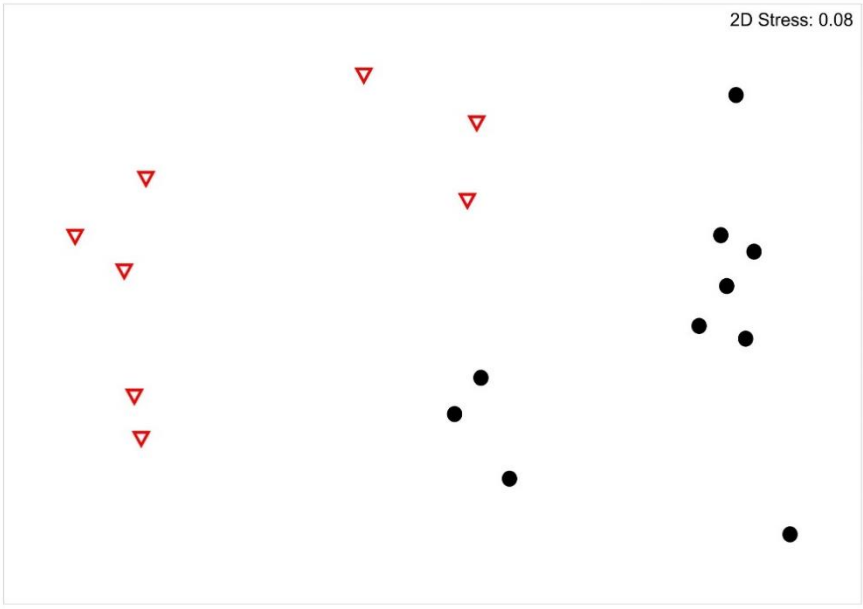
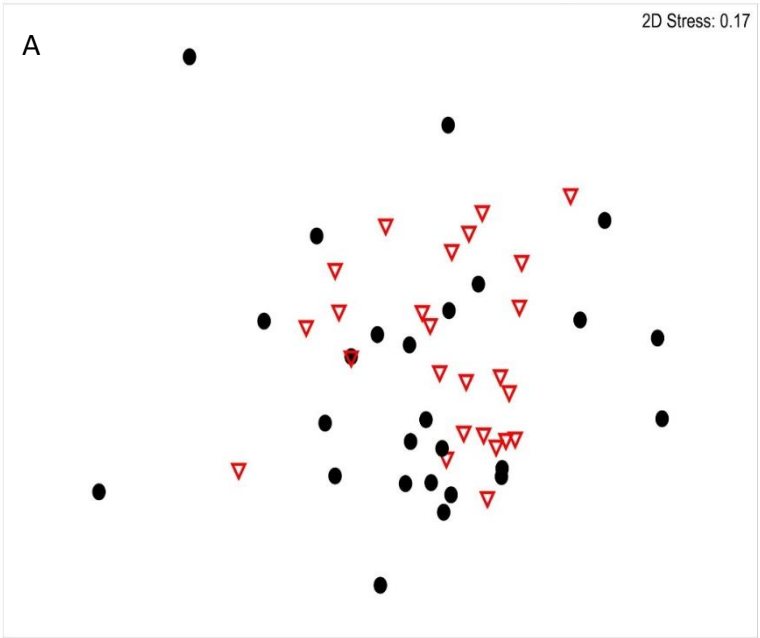


Figure 2.10: Nonmetric multidimensional scaling (MDS) plot showing Gammaproteobacteria communities from mature and immature leaves of *Pseudowintera colorata.* Mature plant●; Immature plant▽.

2.3.2.5 Total fungi

All the factors and their interactions except for the interaction between location and maturity influenced the fungal communities (PERMANOVA, P≤ 0.005) (Table 2.4). The combined tissue data showed that the immature plants formed closer clusters with mature plants being more scattered (Fig. 2.11a). The leaves of the immature plants clustered together while the mature leaves were scattered (Fig. 2.11b). Stems were richer in fungi (n=12) than the roots (n=9) and leaves (n=7) (LSD, *P*≤ 0.05) (Table 2.5).



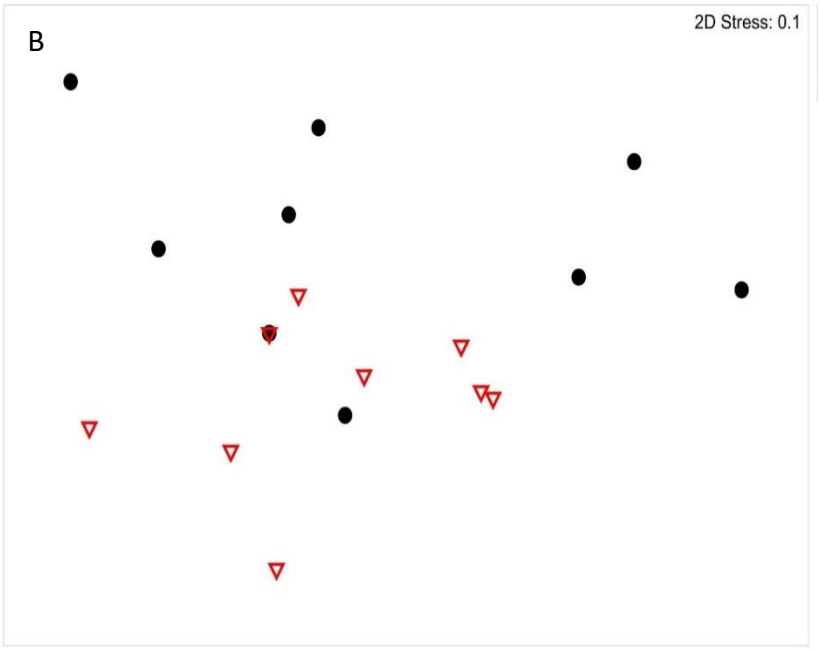


Figure 2.11: Nonmetric multidimensional scaling (MDS) plot showing fungal communities from (a) all tissues of mature and immature plants, and (b) mature and immature leaves of *Pseudowintera colorata*. Mature plant●; Immature plant▽.

2.4 Analysing the structure of *P. colorata* bacterial and fungal endomicrobiome using Illumina MiSeq metabarcoding

Illumina MiSeq was used to analyse the bacterial and fungal endomicrobiome of *P. colorata*. The data from the sequencing of fungi was not of acceptable quality and did not pass the quality filtering during QIIME analysis and thus was excluded from further analysis.

The analysis from the bacterial sequencing returned 6.9 GB raw data. After quality filtering and removing chloroplast and mitochondria DNA from the total sequences (n=1,599,155), 98.9% of total reads were removed (n=1,581,466) (Appendix A.9). A total of 17,689 reads remained with a median value of 871 per sample (minimum= 124, maximum= 20467). An average of 1379 (minimum= 124, maximum= 4308), 3159 (minimum=185, maximum =11501), 8711 (minimum=1637, maximum =20467) reads were obtained from the leaves, stem and root samples, respectively. The reads clustered into 144 OTUs with an average of 8, 9 and 21 OTUs obtained from leaf, stem and root samples of *P. colorata*, respectively. There were several OTUs that appeared in all tissues. From the non-rarefied data, 55.8% of the total leaf OTUs and 51.2% of the stem OTUs were also found in the root samples (Fig. 2.12).

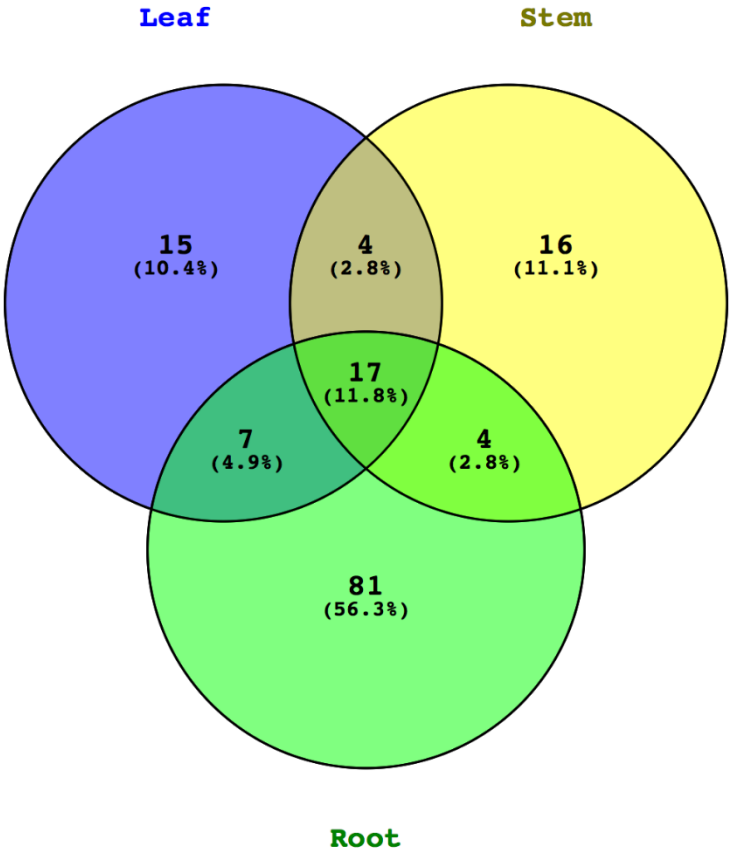


Figure 2.12: Venn diagram showing endophytic bacteria OTUs in different plant tissues of *Pseudowintera colorata*. The total observed OTUs from QIIME were processed in VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html) to show individual and shared OTUs among *P. colorata* plant tissues.

Read numbers per samples was rarefied to 124 (smallest read number) to determine the bacterial community composition in *P. colorata* at phylum and class level (Fig. 2.13). The phylum Proteobacteria was abundant in all tissues irrespective of the location (97.6%). Phyla that were found less abundantly belonged to Actinobacteria (1.2%), Tenericutes (0.7%) Firmicutes (0.1%), Acidobacteria (0.1%) and Bacteroidetes (0.1%) (Fig. 2.13A).

At the class level, Gammaproteobacteria was the most abundant class (89.1%) followed by Alphaproteobacteria (10.0%), Actinobacteria (1.12%) and Betaproteobacteria (0.7%). Less abundant classes were Acidobacteria (0.1%), Bacilli (0.1%), Clostridia (0.05%), Bacteroidia (0.05%) and Saprospirae (0.05%) (Fig. 2.13B). Actinobacterial phyla were only identified in the root samples (Fig 2.13A).

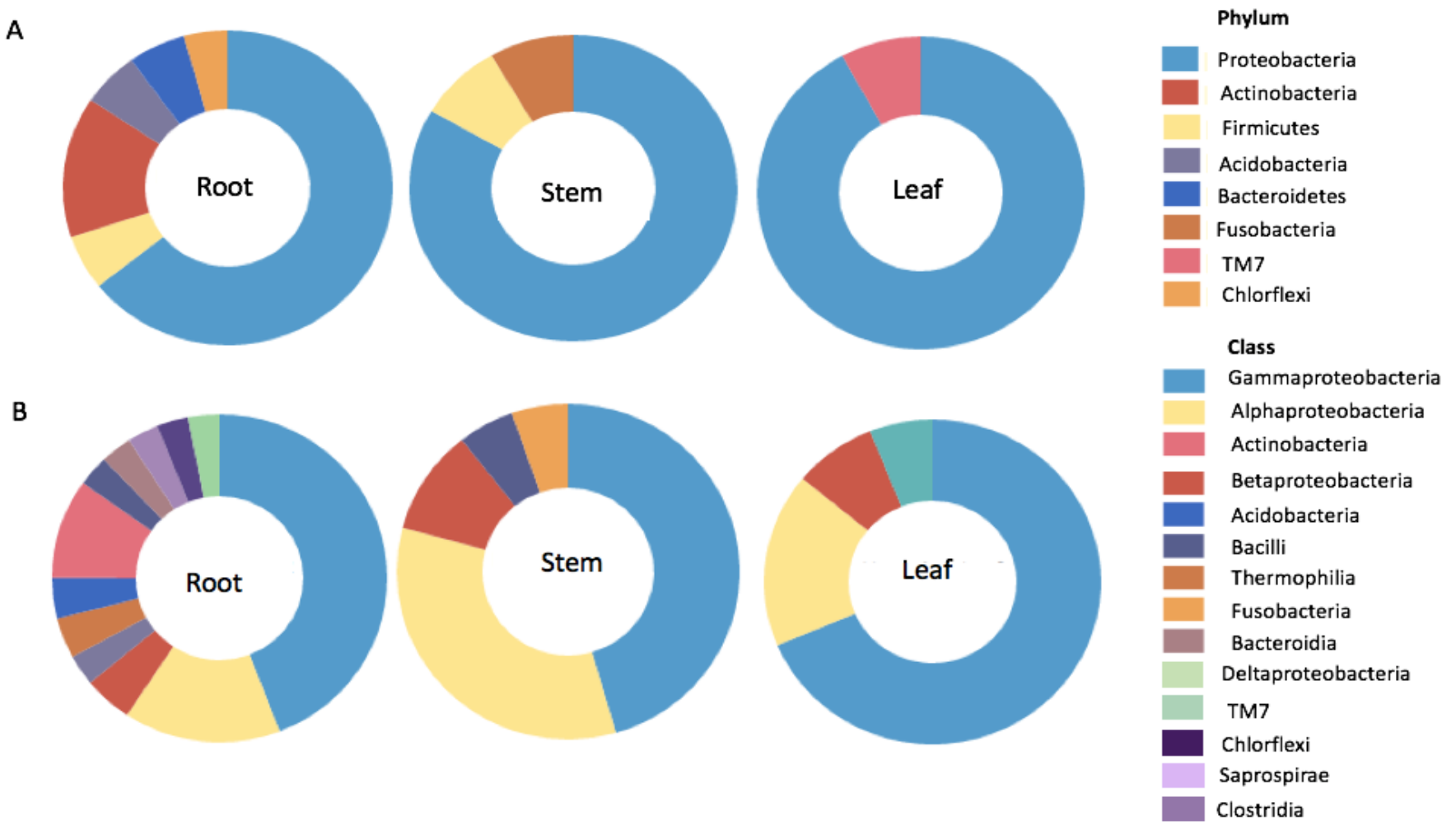


Figure 2.13: Community structure of endophytic bacteria in different plant tissues of *Pseudowintera colorata* as shown by Illumina MiSeq 16S rRNA amplicon sequencing at A) Phylum and B) class level. Donut graphs developed after entering BIOM generated from QIIME and visualised in PHINCH open-source framework.

Two OTUs (Greengenes ID: 646549 and 138914) were found in ≥ 75% of all *P. colorata* leaf, stem and root samples. Both the OTUs belonged to the genus *Pseudomonas* and were identified as the members of the *P. colorata* core endomicrobiome.

Tissue type strongly influenced the richness, diversity and community structure of bacterial endophytes in *P. colorata*. The alpha diversity showed differences in bacterial richness among *P. colorata* tissues. The richness differed in above ground (leaf and stem) and below ground (root) tissues (leaf vs stem, *P*=0.043; leaf vs root, *P*= 0.009; stem vs root, *P*=0.002) (Fig. 2.14).

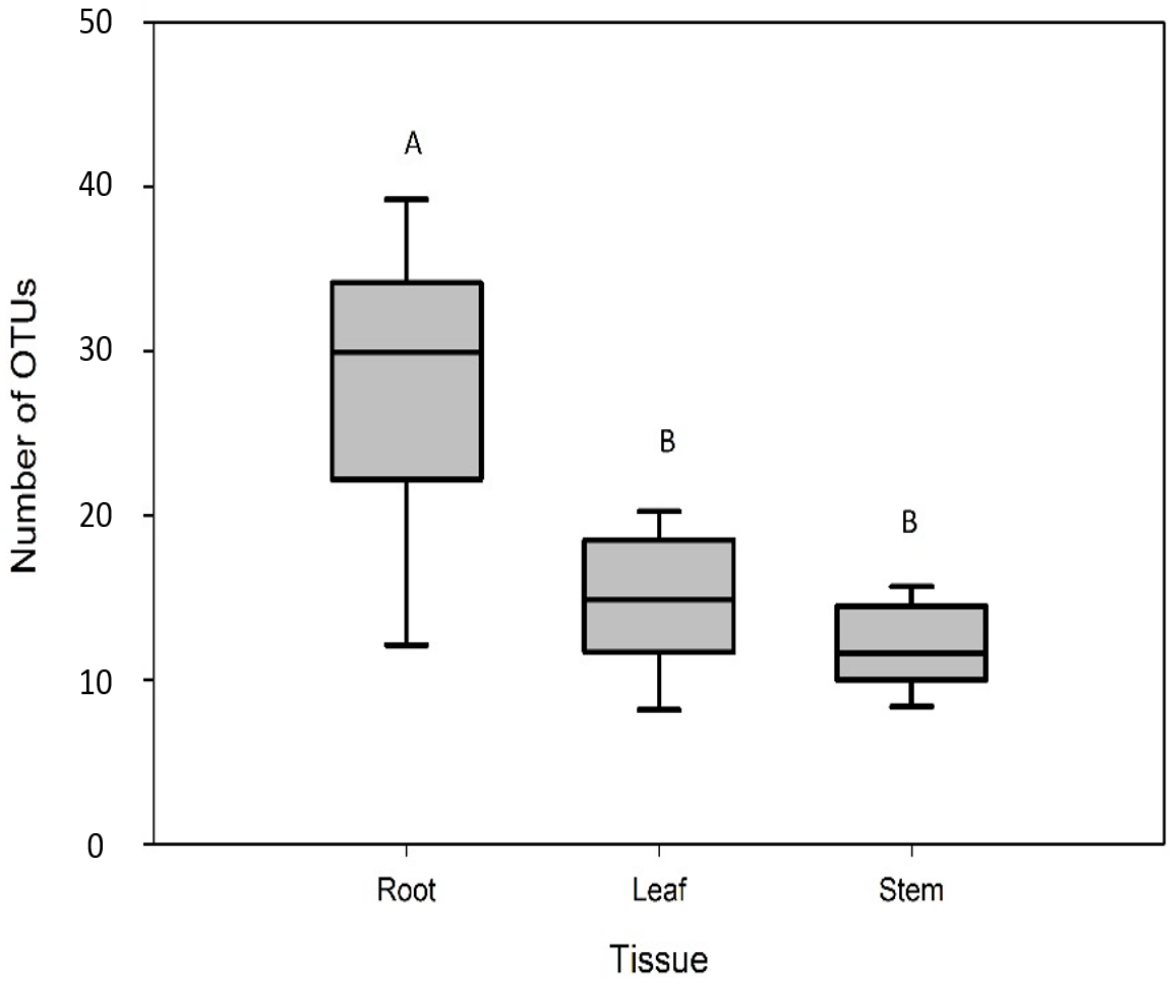


Figure 2.14: Box plot showing the number of observed OTUs of endophytic bacteria in different tissues of *Pseudowintera colorata*. The letters on the bar indicate significantly different number of OTUs between the tissue types at $P \leq 0.05$ as determined by LSD.

Based on the weighted UniFrac analysis, the results showed that plant tissue affected the composition of endophytic bacterial communities (PERMANOVA, *P*=0.001). The bacterial communities clustered based on the plant tissue, with the leaf and stem communities clustering together while the root communities formed a separate cluster (Fig. 2.15).

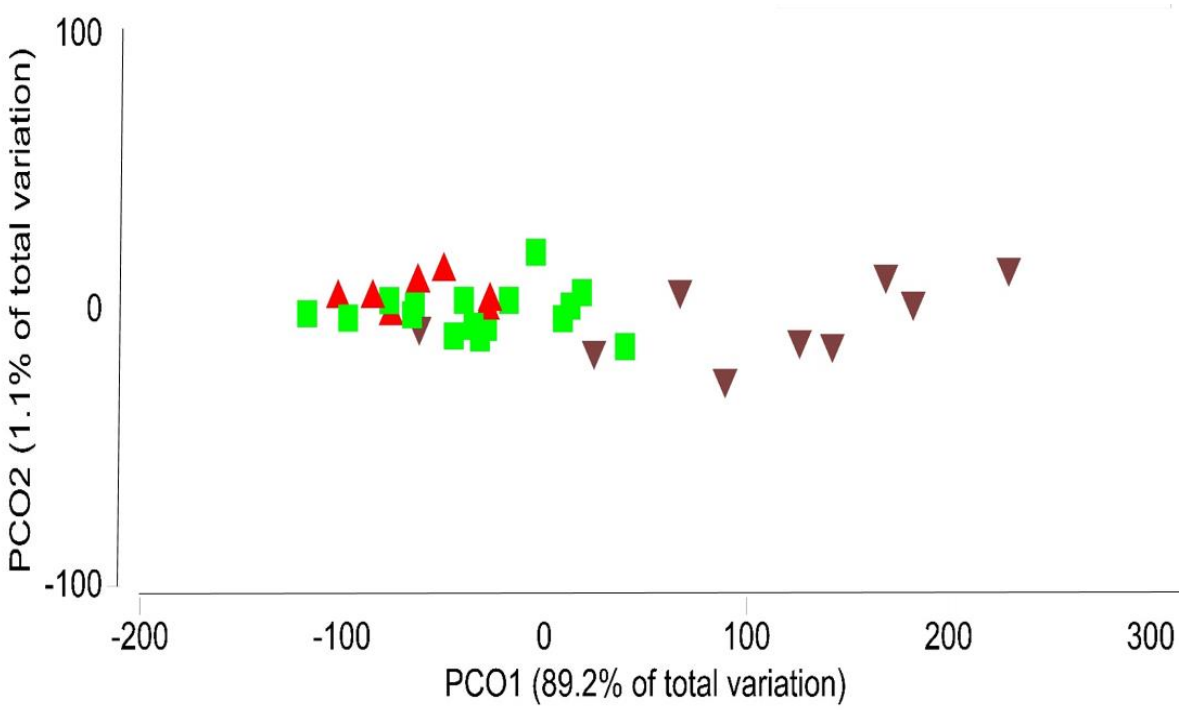


Figure 2.15: Principal coordinates showing similarities between communities of bacterial endophytes from different tissues in *P. colorata*. Leaf (■), Stem (▲), Root (▼).

2.5 Prediction of the function of endophytic bacteria in *P. colorata* using PICRUSt

PICRUSt was used to predict the function of bacterial endophytes in *P. colorata* tissues. According to PICRUSt, 29 level 2 KEGG orthology groups were represented by the dataset. Comparison of the predicted functional gene within the tissues of *P. colorata* revealed that 12 of the 29 gene families were statistically different (LSD, $P \leq 0.05$) (Fig. 16). Gene functions associated with metabolism of co-factors and vitamins, metabolism of carbohydrates, lipids and amino acids, cell motility, signal transduction and poorly characterized were significantly different within the tissues of *P. colorata* (LSD, $P \leq 0.05$). Owing to the antimicrobial properties associated with *P. colorata*, special attention was placed on the gene functions associated with the

biosynthesis of secondary metabolites. PICRUSt analysis revealed that 3.6% of the genes in total relative abundance were associated with the biosynthesis of secondary metabolites (Fig. 2.16).

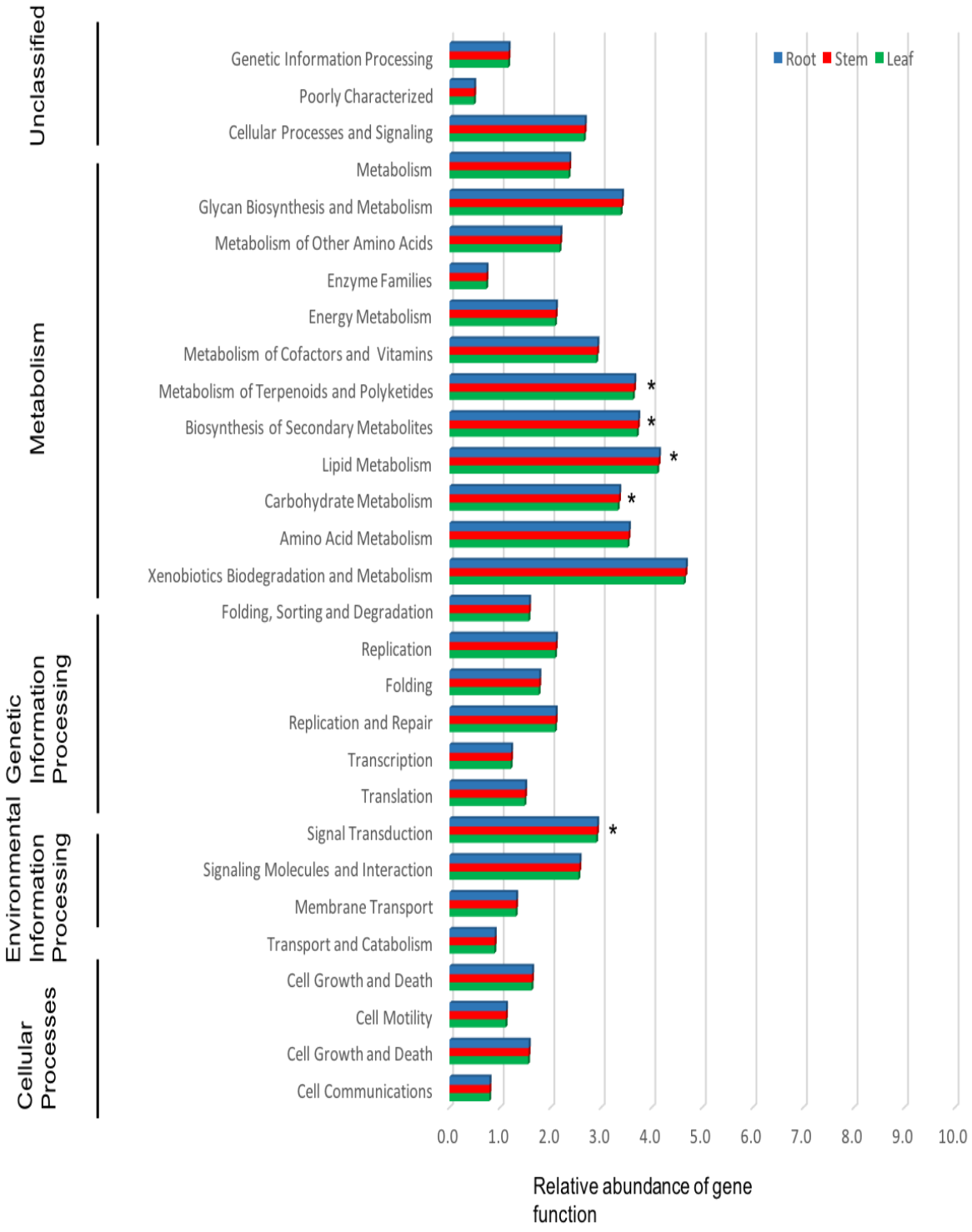


Figure 2.16: Predicted functions (level 2 KEGG orthology group) of the endophytic bacteria in different plant tissues of *Pseudowintera colorata.* Asterisk indicates gene functions that are significantly different (LSD, $P \leq 0.05$).

2.6 Discussion

This is the first study to characterize the structure and diversity of the bacterial and fungal endophytic communities in the roots, stems and leaves of *P. colorata* sampled from ten sites throughout New Zealand. Identifying the community of micro-organisms that inhabit *P. colorata* is a first step towards understanding their function *in planta*. To undertake this work the culture independent molecular tools DGGE and NGS were used.

In this study, the sites that were chosen were part of the New Zealand Department of Conservation (DOC) estate. Thus, the plants were growing in pristine natural environments and not influenced by urbanisation. This was important as several studies have identified urbanization as a major cause of loss of biodiversity (Yan *et al*., 2016, Reese *et al*., 2016). Research by Matsumura and Fukuda (2013) compared the diversity of fungal endophytes in the leaves of nine tree species from three sites (1 suburban forest and 2 rural forests) in Japan to study the effects of forest fragmentation and change in tree species composition following urbanization on fungal endophytic communities. Their results revealed that there was a decrease in the diversity of endophytic fungi in suburban forests compared to rural forests and that the frequency of isolation of endophytes and host specific fungi was lower for the suburban forests.

In this study, the 10 sites sampled across New Zealand covered the West coast, East coast, Central regions of the South Island and the Central region of the North Island. A similar study on another New Zealand medicinal plant, *L. scoparium,* by Wicaksono *et al.* (2016) sampled three sites in the South Island of New Zealand and showed evidence of a core endophytic bacterial community. However, in the current study, inclusion of more sites across the North Island would have been beneficial and enabled the comparison of similarities and differences in *P. colorata* endomicrobiome from both the islands.

This study used DGGE as a molecular tool to analyse the structure of the bacterial and fungal endophytic communities across a large number of sites, plants and tissues (n=87). Although considered an older technique DGGE had advantages and disadvantages for this type of study. DGGE is a very cost effective method that can provide an overview of microbial communities and identify the most significant areas to pursue using NGS (Cleary *et al.,* 2012). In addition, DGGE provides sequences that can be subjected to further

analysis (Valaskova and Baldrian, 2009; Muyzer *et al*., 1993). The disadvantages of DGGE include variations from gel to gel, co-migration of different taxa in the same band, and visualisation of only the more abundant taxa (Muyzer *et al*., 1993; Dowd *et al.,* 2008). Although improved resolution of taxa is possible using taxon specific primers such as α, β and γ Proteobacteria groups used here (Mühling *et al.,* 2008), a key disadvantage of DGGE is that without excising and sequencing all bands, there is no way to ascertain if the band actually comes from the target organism and this may be critical to the outcome of the study.

Analysis of all 10 sites by DGGE showed that tissue type was the main factor influencing the composition and richness of both bacterial and fungal endophytes, suggesting it is an overriding factor in the formation of microbial communities *in planta*. Stems of *P. colorata* had the highest richness of Actinobacteria, Betaproteobacteria and total fungi. Gammaproteobacteria were the richest in the roots, while leaves had the highest richness of Alphaproteobacteria. These results confirmed previous work showing tissue type as a main factor influencing the diversity and richness of endophytic microbes in plants (Jin *et al.,* 2014; Wicaksono *et al*., 2016). Nimnoi *et al.* (2010) studying the endophytic actinobacteria in the roots of *A. crassna* showed similar results suggesting that the host selection plays a critical role in the selection and establishment of the internal endomicrobiome.

Wicaksono *et al.* (2016) showed that plant maturity was a second main factor that affected the community structure of bacterial endophytes in *L. scoparium*. Analysis of plant maturity as a factor influencing the microbiome structure in *P. colorata* was also investigated for a subset of three sites out of the total 10 sites sampled. Because of the difficulty in obtaining plants with a range of maturities in the same sampling area (+/- 20 m radius), the effect of maturity on the endophytic community structure and richness could only be assessed for this subset of three sites where plants of different maturities were present. These results can be considered indicative of the effect of tissue maturity on the endomicrobiome of *P. colorata*. Analysis of additional sites and sampling plants representing different age groups from sites across New Zealand are needed for more robust analysis of the effect of *P. colorata* tissue maturity on the structure and richness of its endomicrobiome. In this study, *P. colorata* plant maturity did not influence the richness

of microbial communities in all the groups for the subset of three sites analysed. The Betaproteobacteria and fungal communities grouped together in the leaves from immature plants, while the leaves from mature plants were more diverse, indicating that there could be a community shift as the plants mature. Results of the study by Wicaksono *et al.* (2016) in the tissues of *L. scoparium*, revealed that tissue maturity did not influence the richness of total bacteria, Beta- and Gammaproteobacteria.

This work reflected that of several other studies that have shown changes in diversity and richness of microbial communities at different stages of plant growth. The study by Wagner *et al.* (2016) on the perennial plant *Boechera stricta* (Brassicaceae), observed a shift in the microbiome composition as the plants age. Da Silva *et al.* (2013) observed that the presence of antimicrobial compounds in the leaves of *Lippia sidoides* influenced the bacterial and fungal communities. Whilst congruent with these examples further research with a greater number of samples is required to gain a clear understanding of the influence of plant maturity on the *P. colorata* endomicrobiome.

For the subset of three sites, the interaction of plant location with maturity influenced the microbial communities across all groups analysed except for Alphaproteobacteria and total fungi. However, additional sites would need to be covered to fully understand the influence of tissue maturity on the microbial communities in *P. colorata.* Wayman *et al*. (2010) and Perry *et al*. (1996) observed that individual plants of *P. colorata* from four different locations had varying chemotypes with key differences in the level of polygodial. Perry *et al*. (1996b) found that *P. colorata* plants from the South Island were of the mixed chemotype with both polygodial and 9-deoxymuzigadial, whereas *P. colorata* plants from the central North Island were predominantly of polygodial chemotype with little or no 9-deoxymuzigadial. Perry *et al.* (1996b) also suggested that the large quantitative variations in the absolute levels of dialdehydes may have been due to sampling leaves of different ages and at different seasons. This variability between locations and polygodial levels in *P. colorata* could contribute to the differences in microbial communities across different locations, or vice versa. A greater number of samples would be required to prove a connection between the diversity and abundance of the microbial communities in *P. colorata* and plant chemistry.

Next generation sequencing methodologies such as the Illumina MiSeq used here detects more species with a greater accuracy and produces significantly more data when compared to DGGE (Yu *et al*., 2015; Qin *et al*., 2016). Using DGGE, Wicaksono *et al*. (2016) showed that the communities of bacterial endophytes were less variable in mature plants of *L. scoparium* compared to the immature plants. Based on the results of that study and the DGGE data showing that mature plants had a different microbiome, only mature *P. colorata* plants were selected for Illumina MiSeq analysis.

The Illumina MiSeq analysis of the 16S region worked and majority of the reads were identified as either chloroplast or mitochondria. The primers for both 16S and ITS2 regions used in Illumina MiSeq analysis in this study amplified chloroplast, plastid and mitochondrial DNA. Analysis of the bacterial data revealed that 98.9% of the total reads were assigned as Cyanobacteria, chloroplast and mitochondrial DNA and removed during filtering. Researchers have indicated that the number of chloroplasts and mitochondria varies depending on the species of plant, cell type and the age of the tissue (Shaver *et al.,* 2006). For example, Shaver *et al*. (2006) revealed that the number of chloroplast DNA copies in the tobacco leaf cells could be as high as 10,000. Similar study by Wicaksono (2016) on *L. scoparium* endophytic bacteria used Illumina MiSeq and during quality, filtering 96.4% of the reads were removed. In a study, using the same primers to analyse the endophytic bacteria in the tissues of *Aloe vera* using Illumina MiSeq, Akinsanya *et al.* (2015) reported that of the total 2,599,551 sequences, 115,792 cleaned sequences remained after removal of chimera, chloroplast, mitochondria and eukaryote sequences. These findings suggest that the number of chloroplasts and mitochondria in the plants are indeed different and influence the output significantly. However, for future studies, the sequencing could be improved by either using refined extraction methods that can specifically exclude plant (chloroplast and mitochondria) DNA (Lutz *et al.,* 2011) or using 16S rRNA primers such as 799F and 1391R (Beckers *et al.,* 2016) to minimize plant DNA amplification. The protocol by Lutz et al. (2011) used Triton X-100, which selectively lyses chloroplast and mitochondria. This modification could be adapted for future studies with the modified CTAB method as used in this study. Beckers *et al.* (2016), used specific mismatch primers which amplified the 16S rDNA of endophytic bacteria while simultaneously avoiding the amplification of chloroplast DNA sequences. In addition, the

authors suggested that instead of using V3-V4 region for plant-associated bacteria, the choice of V6-V7 would be appropriate with these primers to avoid co-amplification of organellar DNA. The authors found that the number of reads that could unambiguously be classified at the phylum level was higher for the V6-V7 region (46%) compared to V3-V4 region (21%). While the Illumina MiSeq worked for the 16S regions, it was not successful for the ITS regions. The PCR for the ITS sequences was carried out using the fITS7 and ITS4 primers for the ITS2 region as described by Gweon *et al.* (2015). The PCR yielded amplicons, which were visualized in agarose gel. However, when sequenced with Illumina MiSeq platform there were no sequences. The PCR products were then checked with Sanger sequencing and it was revealed that only 50% of the samples generated sequence data. The PCR was also carried out for the ITS1 region, which provided an outcome similar to that for the ITS 2 region. Following the Illumina MiSeq run, the data received did not pass the quality filtering after the paired ends were joined in QIIME. This was not pursued further due to time constraints.

As the tissues of *P. colorata*, especially the leaves, were rich in polyphenolic compounds and antimicrobial compounds (polygodial and 9-deoxymuzigadial), the process of extracting DNA was challenging. Using the commercial kits (PowerPlant by MoBio, DNeasy Plant Mini Kit and Gentra Puregene Tissue Kit by Qiagen, and Quick-DNA Plant/Seed Miniprep Kit by Zymo Research) extracting DNA was not successful, with the kits yielding less than 1 ng per sample. In contrast, the modified CTAB method by Allen *et al.* (2006) used in this study yielded up to 35 ng of DNA for some samples, which was of good quality and was sufficient for Illumina MiSeq.

Illumina MiSeq analysis of the 16S rDNA sequences revealed that roots were composed of 55.8% and 51.2% of leaf and stem OTUs, respectively, indicating that the roots harbour a large reserve of endophytes. This could be because roots contact the soil and are in constant interaction with the rhizosphere microbial communities (Long *et al.,* 2008). Roots are also naturally wounded by insects feeding on them, emergence of lateral roots etc. that may provide entry points (Hallmann, 2001). The relative richness of the roots could also be attributed to the absence of antimicrobial compounds (Compant *et al*., 2010; Edwards *et al*., 2015; Hardoim *et al.,* 2008). Active microbiome acquisition by the plant could also be a reason for the roots being relatively rich in endophytes (Edwards *et al.,* 2015). Research

groups have suggested that plants assemble their root microbiome by 2 steps: 1) general recruitment around the roots, and 2) entry inside roots, which involves species-specific genetic factors (Edwards *et al.,* 2015; Bulgarelli *et al.*, 2013). Root exudates have been indicated as a major reason for the acquisition of members of the microbiome from soil, while the leaves and stems have little surface soluble organic compounds thus accounting for the differences in community abundances (Bulgarelli *et al.*, 2013).

Only approximately half of the bacterial root endophytes were also found in aerial parts of the plant. Other research has shown that not all the acquired endophytes can migrate through the complex plant system and cells to colonize the aerial plant tissues (Hallmann, 2001). Successful colonization by endophytes also depends on the host plant, with some capable of surpassing the host defences better than the others (Ryan *et al.,* 2008). Compant *et al.* (2005b) studied the patterns of colonization in *Vitis vinifera* L. cv. Chardonnay plants by *Burkholderia* sp. strain PsJN and found that the cell wall degrading endoglucanase and endopolygalacturonase secreted by strain PsJN helped the bacterium enter internal root tissue. Hallmann *et al.* (2001) found that bacteria moved within root hairs and colonized the epidermal cells in high numbers but the adjacent cells were free of intercellular colonization. This might account for the unshared OTUs found only in the roots. Leaves and stems of *P. colorata* also had some unshared OTUs. The natural routes of entry like stomata and hydathodes in leaves and lenticels on stems may allow specific microbes from the environment and phyllosphere to enter the tissues (Hallmann, 2001). Apart from these natural routes other biotic and abiotic factors like herbivory, pathogens and environmental causes like hail and rain can cause damage to the tissues aiding in their penetration (Hallmann, 2001; Van der Putten *et al.,* 2007). Endophytes in the aerial parts of the plant must also tolerate rapid changes in temperature, humidity and exposure to UV radiation (Redford *et al.,* 2010; Whipps *et al.,* 2008). Finally, the presence of antimicrobial compounds in the leaves of *P. colorata* (Perry and Gould 2010; Perry et al., 1996; Mc Callion *et al.,* 1982) could also act as selective pressure influencing the overall microbial diversity.

Illumina MiSeq analysis revealed that 89.1% of the total reads belonged to Gammaproteobacteria class, particularly to the genus *Pseudomonas*, making them the most abundant group in endomicrobiome. Members of Gammaproteobacteria are relatively common endophytes found in plants (Germaine *et al.,* 2004). Two OTUs

belonging to the genus *Pseudomonas* were identified as the members of *P. colorata* core endomicrobiome as they were present in at least 75% of samples. A similar observation was found in another native New Zealand medicinal plant *L. scoparium*, with Gammaproteobacteria being the most significant group and a *Pseudomonas* sp. member identified as the core endomicrobiome (Wicaksono, 2016). The definition of the "core endomicrobiome" is variable within the literature with some research groups defining it as the OTUs present in at least 50% of the samples, with others at 90%. Research by Sánchez-López *et al*. (2017) revealed *Methylobacterium* as the dominant OTU in the core microbiome of *Crotalaria pumila* seeds and constituted more than 80% of the core microbiome. The genus *Pseudomonas* is ubiquitous in nature (Berg *et al.,* 2011) and part of the core endomicrobiome of many plants ranging from model plants like *Arabidopsis thaliana* to medicinal plants like *Cannabis sativa* (Bragina *et al.,* 2012; Lundberg *et al.,* 2012; Müller *et al.,* 2015a). *Pseudomonas* sp. can confer unique characteristics to the host plant and are well known for plant growth promotion (Mercado-Blanco and Bakker, 2007; Long *et al.,* 2008). As with DGGE, the results of Illumina MiSeq analysis also confirmed that plant tissues affected the composition, diversity and richness of endophytic bacteria in *P. colorata*.

The endomicrobiome may be involved in providing indirect protection against pathogens (Bulgarelli *et al.,* 2013), and may be involved in the host biochemical pathways by contributing to the production of secondary metabolites. PICRUSt was used to assign putative functions to the endophytic bacteria in *P. colorata*. PICRUSt is a tool that uses the existing database of microbial genomes to predict the gene content of a microbial community 16S rRNA gene (Langille *et al*., 2013). For each 16S rRNA gene, PICRUST retrieves the last phylogenetic common ancestor with known sequenced genome and predicts the functional gene content of the 16S rRNA marker gene, which represents the unknown genome (Langille *et al.*, 2013). The limitations of PICRUSt are that it cannot predict the functional genomes of eukaryotes and that it does not reflect variations at the strain level and is biased by its dependency on hypervariable regions (V1-V9) of the 16S rRNA gene (Langille *et al.*, 2013; Smith *et al*., 2014). PICRUSt was applied to the data to gain some information about the potential functional roles of the bacterial endophytes of *P. colorata.* Kawasaki *et al*. (2016) used PICRUSt to assess the functional gene content of the

bacterial communities colonizing *Brachypodium distachyon* seminal and nodal roots and found gene categories related to metabolism, genetic information processing, cell motility and membrane transport. Sánchez-López *et al*. (2017) used PICRUSt in their analysis and found that the core microbiome of *Crotalaria pumila* seeds presented genes related to metabolism (amino acid, carbohydrates, and lipids), cellular processes, and energy metabolism.

PICRUSt analysis showed that some of the endophytic bacteria of *P. colorata* may be involved in the production of bioactive secondary metabolites. Comparing the predicted gene functions using PICRUST between different plant tissues of *P. colorata* it was revealed that the endophytic bacteria in the tissues were associated with different metabolic activities like metabolism of carbohydrates and amino acids, which could help with penetration of root cell walls and aid in colonization (Compant *et al*., 2010; Krause *et al*., 2006). Yuan *et al*. (2016) used PICRUSt to study the halotolerant plant *Suaeda salsa* (seepweed) microbiome and found that endophytic bacteria associated with *S. salsa* had functional gene categories related to salt stress acclimatization, nutrient solubilisation and competitive root colonization.

To summarize, this study is the first to describe the structure of the endomicrobiome of the New Zealand native medicinal plant *P. colorata*. A core endomicrobiome that is tissue specific was revealed in the mature tissues. The identification of a core endomicrobiome suggests that the endophytes of *P. colorata* are likely to be important and involved in the physiological processes of the host plant.

Chapter 3

Bioactive potential of endophytic bacteria and fungi inhabiting *Pseudowintera colorata*

3.1 Introduction

Endophytes, in return for nutrients and habitat, may provide several benefits to the plant, which include growth promotion, biological control of phytopathogens, and tolerance against biotic and abiotic stress (Bush *et al.,* 1997; Hallmann *et al.,* 1997; Schutz, 2001; Hardoim *et al.,* 2008). Endophytes can prevent colonisation of phytopathogens by competing for niches, releasing enzymes and antimicrobial compounds (Strobel and Daisy 2003; Berg and Hallmann, 2006). Endophytes can also activate the host defence mechanism against pathogens through induced systemic resistance (ISR) (Kloepper and Ryu, 2006). Plant growth promotion is brought about by mechanisms such as secretion of siderophores, nitrogen fixation, and solubilisation of phosphorus (Richardson *et al.,* 2009). Endophytes can also increase the fitness of the host plant by conferring anti-herbivore alkaloids and enhancing photosynthesis (Sanchez-Azofeifa *et al.,* 2012; Gundel *et al.,* 2012).

There is a lack of significant knowledge about the endophytes of New Zealand native plants, including those with medicinal uses, with the only major study recently published by Wicaksono *et al.* (2016) on *L. scoparium*. *Pseudowintera colorata* (horopito) has been an integral part of traditional Maori medicine (rongoā) and has been used in the treatment of fever, toothache, skin infections and gonorrhoea (Brooker et al., 1987). Mc Callion *et al.* (1982) identified the main biologically active chemical constituent of *P. colorata* as the sesquiterpene dialdehyde, polygodial. Gerard *et al.* (1993) later reported that in addition to polygodial, another sesquiterpene dialdehyde 9-deoxymuzigadial was found in high concentrations in *P. colorata* and had antifeedant properties. Polygodial possesses anti-fungal and anti-bacterial properties (Kubo *et al*., 2001; Kubo *et al*., 2005) along with insect antifeedant properties (Gerard *et al.,* 1993).

International research has shown that the interactions between endophytes and their host plants contributes to the co-production of bioactive molecules (Heinig *et al.,* 2013). Wicaksono *et al.* (2016) showed that endophytic bacteria recovered from New Zealand native medicinal plant *L. scoparium* solubilized phosphate, produced siderophores and

showed antifungal activity against phytopathogenic fungi *Neofusicoccum luteum* and *Ilyonectria liriodendri*. Studying endophytic microbes from medicinal plants as a potential source of new bioactive compounds is an area of increasing interest worldwide and a new area of research in New Zealand.

The endophytes associated with *P. colorata* might also play a role in the production of polygodial and be involved in the plants biochemical pathways such as growth and protection against phytopathogens. PICRUSt analysis from the previous chapter suggested that the endophytic bacteria of *P. colorata* may biosynthesise secondary metabolites. This is the first study to describe the bioactive potential of the endophytic bacteria and fungi inhabiting *P. colorata*.

The main objectives of this chapter are to assess:

1) The bioactive potential of bacterial and fungal endophytes of *P. colorata* against phytopathogenic fungi and bacteria
2) Ability of the endophytes to inhibit human pathogenic bacteria and yeasts
3) Ability of endophytic bacteria and fungi to solubilize phosphate and secrete siderophores

3.2 Materials and Methods

3.2.1 *Sampling locations* – *Pseudowintera colorata* tissues that were sampled from locations described in Section 2.2.1 were also used for recovering culturable endophytic bacteria and fungi.

3.2.2 *Plant sampling* – The leaves, stems and roots of *P. colorata* were sampled for the study. Since *P. colorata* is a very slow growing plant and the age of the sampled plants was unknown, the plants were classified as mature, intermediate and young plants based on their height. Mature plants (> 3 m), intermediate plants (< 3 m > 1 m) and young plants (</= 1 m).

3.2.3 *Sample processing* – The tissues that were collected in Section 2.2.3 were washed with tap water to remove soil and other debris and dried on a clean paper towel for 30-45 s. The tissues were surface sterilized with 96% ethanol for 10 s, followed by 2.5% freshly prepared sodium hypochlorite for 3 min and three consecutive washes with sterile distilled water for 1 min each. The tissues were cut into 1 mm width slivers using a sterile scalpel

and five to six sections from three tissues were plated onto R2A agar (Difco) and synthetic nutrient agar (SNA; SIFIN) for recovery of endophytic bacteria and fungi respectively. Prior to plating, a leaf imprint was taken to ensure that the sterilization process was effective and there were no contaminants remaining on the tissue surface. Also 100 µL of the wash water from the final sterile distilled water rinse after each sterilization process was plated to ensure the process was effective.

3.2.4 Isolation and preservation of bacterial and fungal endophytes from *P. colorata*

For isolating bacterial endophytes, 5-6 pieces of surface sterilized tissue slivers (leaves, stems and roots) were plated onto R2A agar which was amended with nystatin (50 µg/mL)(Lee *et al.,* 2014) to prevent the growth of fungi and allow selective isolation of bacteria. The plates were sealed and incubated at 25°C. The plates were observed daily for 3-5 d to identify bacterial colonies emerging from the tissues. Emerging bacterial colonies were sub-cultured onto nutrient agar (NA, Difco) plates and streaked to isolate single colonies. A single colony was then inoculated into a 1.7 mL tube containing 500 µL sterile nutrient broth (NB, Difco) and incubated in a shaker incubator (Labnet 211 DS) at 25°C and 150 rpm for 24 h. After 24 h, 500 µL sterile glycerol (40%) was added to the tubes and vortexed to mix the broth and glycerol. The tubes were stored at -80°C for future work.

For the isolation of fungi, SNA plates were amended with ampicillin (100 µg/mL). The surface sterilized plant tissues described in Section 3.2.3 were plated and the plates incubated at 20°C in a 12 h light/12 h dark cycle. The plates were observed to look for mycelium emerging from tissues. The mycelium was transferred to sterile potato dextrose agar (PDA, Difco), and the plates were incubated at 20°C for 5-7 d. After the colony covered more than half the plate, 6 mm mycelial plugs were taken from the margins of the colony. The plugs (6 to 8) were placed in a sterile 2 mL screw cap tube containing sterile fungal preservation media (Appendix B.1). The tubes were stored at -80°C.

3.2.5 Functional activity of endophytic bacteria isolated from *P. colorata*

3.2.5.1 Activity against phytopathogenic fungi

***Dual culture assay*-** To assess the activity of endophytic bacteria isolated from horopito as potential biocontrol agents, they were screened against phytopathogenic fungi *Neofusicoccum luteum* ICMP 16678*, N. parvum* MM562*, Ilyonectria liriodendri* WPA1C and

Neonectria ditissima ICMP 14417. The assay was carried out on Waksman agar (WA, Appendix B.2) plates. All phytopathogenic fungal isolates were obtained from the Lincoln University Plant Microbiology culture collection, apart from *N. ditissima* ICMP 14417 which was obtained from Landcare Research, New Zealand. Bacterial strains were revived by inoculating a loop full of culture from the culture tubes in cold storage (-80°C) onto WA and incubating at 25°C for 24-48 h. For phytopathogenic fungi a portion of stored mycelial plug was plated face down WA plates. The assays were performed with a 24-48 h old culture.

For the assays, a 6 mm disc from the edge of a fungal colony was placed in the centre of the test plate. Using a sterile inoculation loop, the bacterial strain was placed 5 cm away from the fungal colony (Fig.3.1). The plates were sealed and incubated at 25°C for 1-4 weeks based on the growth rate of the fungal colony towards the bacterial strain. The plates were observed daily and the measurements of the fungal colony towards the bacterial isolate was recorded in comparison to an un-inoculated fungal control plate.

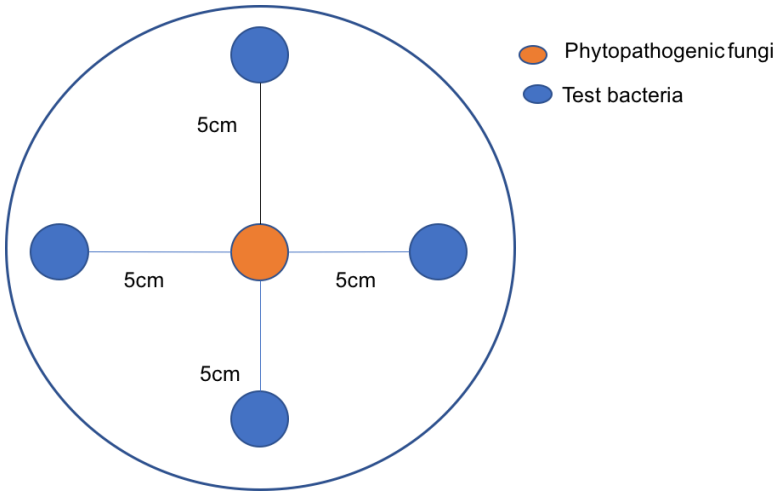


Figure 3.1: Depiction of assay procedure testing phytopathogenic fungi against endophytic bacteria isolated from *P. colorata.*

3.2.5.2 Activity against phytopathogenic bacteria

Dual culture assay **–** The endophytic bacteria isolated from *P. colorata* were also screened against the phytopathogenic bacteria *Pectobacterium atrosepticum* and *P. brasiliensis* causing potato tuber soft rot. Pure cultures of *P. atrosepticum* and *P. brasiliensis* were obtained from Abigail Durrant and Dr Andy Pitman at the Bioprotection Research Centre

(BPRC) in Lincoln University. The test pathogens were grown overnight in nutrient broth and 100 μL was spread onto WA plates using a sterile spreader to create a bacterial lawn. The endophytic bacterial test strains were then inoculated using a sterile loop. The plates were sealed and incubated at 25°C and observed daily. Presence of a clear zone around the bacterial strain was noted as positive and the results were recorded in comparison to an uninoculated phytopathogenic bacterial control plate.

3.2.5.3 Activity against opportunistic human pathogenic bacteria and yeasts

The opportunistic human pathogens used in this study were obtained from The Institute of Environmental Science and Research (ESR), Porirua, New Zealand. The strains selected were bacteria *Staphylococcus aureus* 297, *Escherichia coli* 916 and yeast *Candida albicans* 3395. The pathogens were purchased as lyophilized vials. The test pathogens were revived by mixing with 200 μL of sterile NB and plated onto NA for *S. aureus* 297 and *E. coli* 916, and PDA for *C. albicans* 3395. The plates were sealed and incubated at 25°C for 48-72 h.

Dual culture assay – Endophytic bacterial isolates were revived from cold storage by streaking onto NA plates using a sterile inoculation loop. The plates were incubated at 25°C for 24 hours. The test pathogens were grown overnight in NB on a shaker incubator (Labnet 211DS) set at 25°C and 150 rpm. After 24 h, 100 μL of the overnight culture was spread onto WA plates using a sterile spreader. From the plates used to revive the endophytic bacteria, a sterile loop was used to pick up a single colony and inoculated onto the test plates. The plates were sealed and incubated at 25°C and were observed daily for 3-5 d. Presence of a clear zone around the bacterial strain was noted as positive and the results were recorded in comparison to an uninoculated control plate.

3.2.5.4 Secretion of siderophores

The ability of the endophytes to secrete siderophores was tested on chrom-azurol S agar (CAS Appendix B.3). The bacterial strains were revived from cold storage as described in Section 3.2.5.1 and a sterile inoculation loop was used to touch a single bacterial colony, which was then inoculated on CAS agar and the plates were incubated at 25°C for 5-7 d. Positive results were indicated by the presence of an orange halo.

3.2.5.5 Determining if the endophytes produced bioactive compounds constitutively or in the response to an antagonist

The isolates for this assay were selected based on the bioactivity against phytopathogenic fungi in the dual culture assays. To understand if the endophytes produce bioactive compounds continuously or if they are induced by the antagonist, the test bacteria were inoculated onto WA plates, as described in Section 3.2.5.1, 48, 24 and 0 h prior to the inoculation of the test pathogen. The phytopathogenic fungi were then plated as described in Section 3.2.5.1. The plates were sealed and incubated at 25°C for 5-7 d. Untreated controls containing only each of the phytopathogenic fungi were set up to compare the normal growth of fungi to the treatments.

3.2.5.6 Production of bioactive compounds in liquid culture

Endophytic bacteria that inhibited bacterial test pathogens (phytopathogenic bacteria and opportunistic human pathogens) in the plate assays were further tested for metabolite production in liquid culture. Using a sterile inoculation loop, a single colony of the bioactive strains were inoculated into a 50 mL tube containing 20 mL sterile Waksman broth (Appendix B.4). The tubes were incubated in a shaking incubator for 5 d at 25°C. After 5 d, the tubes were harvested and the cultures were centrifuged at 20,000 X *g* for 15 min to pellet the cells. The supernatant was filtered using a 0.22-micron pore size filter. Test pathogens were grown overnight in NB incubated at 25°C. After 24 h, 100 µL of the overnight culture was spread onto WA plates using a sterile spreader. Using a sterile 6 mm cork borer, a well was made in the middle of the plate. To this well, 100 µL of the cell free supernatant was added. The plates were prepared in triplicates. For the control plates, the wells were inoculated with sterile NB. The plates were sealed and incubated at 25°C and were observed daily for 5-7 d. Presence of a clear zone around the well was noted as positive and the results were recorded in comparison to a control plate.

3.2.6 Functional activity of endophytic fungi isolated from *P. colorata* against *C. albicans*

From the endophytic fungi (n=200) isolated from *P. colorata*, 50 representative isolates were selected for screening against *C. albicans*. The assays were carried out on WA. The assay protocol from Liu *et al.* (2010) was modified and adapted. GelAir Cellophane (Biorad) membranes were cut using a scalpel and a Petri dish lid as reference. The cellophane

membranes were then placed between filter papers in a beaker and autoclaved. The membranes were then carefully transferred onto the WA plates using sterile forceps. The membrane was gently pressed onto the agar using a sterile spreader to ensure that the membrane adhered to the agar. The fungi were revived from -80°C onto PDA plates and incubated at 20°C. After the fungal colony covered about half of the plate, using a sterile cork borer a 6 mm plug from the margins of the colony was transferred onto the centre of the cellophane membrane on the WA plates (Fig. 3.2). The plates were then sealed and incubated for 7 d at 20°C. After 7 d, the cellophane membranes with the fungal mycelium were carefully lifted using sterile forceps and discarded.

Using a sterile spreader, 100 µL of the overnight culture of *C. albicans* grown in NB was spread onto the test plates (Fig.3.2). The plates were sealed and incubated at 25°C and were observed daily. Presence of a clear zone on the plates was noted as positive and the results were recorded in comparison to an uninoculated *C. albicans* control plate.

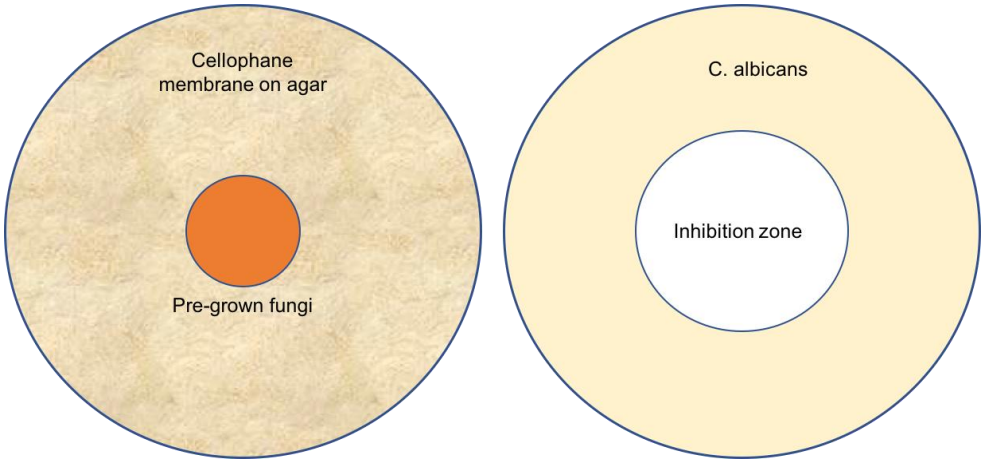


Figure 3.2: Diagram depicting the plate set up for the assay of endophytic fungi against *Candida albicans.*

Strains that showed activity against *C. albicans* were screened further for production of metabolites in liquid culture.

3.2.7 Production of bioactive compounds by fungi in liquid culture

To test the effect of secreted metabolites on the test pathogens, 6 mm plugs of the bioactive strains identified from the dual culture assays were grown in flasks containing 150 mL of Waksman broth for 7 days at 25°C in a shaking incubator. After 7 days the flasks were harvested and the cultures were centrifuged at 20,000 X *g* for 15 min to pellet the mycelia. The supernatant was then filtered using a 0.22-micron pore size filter. The

resulting supernatant was then incorporated into autoclaved WA cooled to 50°C to get 1% and 10% (v/v) filtrate incorporated agar and also used for assays against bacterial pathogens. Three plates per treatment per fungi were set up and inoculated with the test pathogen plugs. Controls of the fungi were grown on unamended WA. After incubation the colony diameter in perpendicular directions was measured and the growth compared to the control.

3.2.8 Identification of culturable bacteria by sequencing the 16S rRNA gene and fungi by sequencing the ITS region

From the bacterial and fungal isolates that showed highest activity within each assay, 5-10 bacteria and fungi were selected to identification by sequencing the 16S rRNA gene and the ITS region, respectively. The DNA for each strain was extracted using the PureGene kit (Qiagen) as per the manufacturer's instructions. Using the primer pairs F27 (5'-AGA GTT TGA TCM TGG CTC AG-3'), R1494 (5'-CTA CGG YTA CCT TGT TAC GAC-3') (Weisburg *et al.,* 1991, Neilan *et al.,* 1997b) for bacteria and ITS1 (5'-TCC GTA GGT GAA CCT GCG G-3'), ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3') (Verma *et al.,* 2011, Linnakoski *et al.,* 2012) for fungi, the 16S rRNA and ITS regions were amplified, respectively. The PCR were carried out in an Applied Biosystems Proflex PCR system in a total volume of 25 μL containing 1 μL of template DNA, 2.5 μL buffer, 200 μM dNTPs, 1 U *Taq* DNA polymerase, 1 μL of each primer (10 μM) and 18.75 μL water for both the 16S rRNA and ITS regions. Positive and negative controls were run for each PCR. The PCR conditions for the 16S rRNA region were as follows: initial denaturation was performed at 95°C for 30 s; followed by denaturation at 94°C for 30 s; primer annealing at 55°C for 30 s; and chain elongation at 72°C for 1 min. These three steps were repeated for 35 cycles. Final elongation was performed at 72°C for 7 min and the reactions were cooled to 4°C. For ITS region, the PCR conditions were as follows: initial denaturation was performed at 94°C for 2 min; followed by denaturation at 94°C for 30 s; primer annealing at 60°C for 30 s; and chain elongation at 72°C for 1 min. These three steps were repeated for 35 cycles. Final elongation was performed at 72°C for 7 min. After the PCR reaction, 4 μL of the product was mixed with 4 μL of loading dye and loaded onto a 1% agarose gel (Bioline, Bioline USA Inc.) in 1x TAE (Appendix A.4) and run for 1 hour at 100 V. Gels were stained in ethidium bromide solution (Appendix A.5) for 15 min and destained in water for 15 min and the gels were visualized under UV light using

the UVIreader (UVItec Ltd, Cambridge, UK). The PCR-amplified 16S rRNA and ITS regions were sequenced directly at the Lincoln University Sequencing Facility. The sequences obtained were trimmed using DNAMAN v4 (Lynnon Biosoft, Canada) to remove ambiguous regions. The sequences were then compared against those of known origin using NCBI BLAST (basic local search alignment tool) and the GenBank database (www.ncbi.nlm.nih.gov).

3.2.9 Detection of antibiotic producing genes from bacteria using PCR

DNA from the endophytic bacteria (n=8) that were extracted in Section 3.2.7 was used for PCR to identify the genes encoding antibiotic production. Three genes encoding the production of antibiotics 2,4 –diacetylphloroglucinol (*phl*D), phenazine (*phz*C) and pyrollnitrin (*prn*C) were amplified using PCR (Table 3.1) (Mavrodi *et al.,* 2001; McSpadden *et al.,* 2001; Mazurier *et al.,* 2009). The bacteria for this assay were selected on the basis of their activity against the phytopathogenic fungi.

The PCR were carried out in an Applied Biosystems Proflex PCR system in a total volume of 25 μL containing 1 μL of template DNA, 12.5 μL DreamTaq Green PCR Master Mix (2X) (Thermo Scientific), 1 μL of each primer (10 μM) and 9.5 μL PCR water. The PCR product was run in agarose gel and visualized as described in Section 3.2.7.

Table 3.1 Primer and PCR conditions used to amplify the three different genes encoding the production of antibiotics from the selected bacteria.

Gene Target	Primer	Sequence	PCR conditions	Product size (bp)	References
*phl*D	B2BF	ACC CAC CGC AGC ATC GTT TAT GAG C	95°C for 3 min, 35 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, 72°C for 5 min	600	McSpadden Gardener *et al.* (2001)
	BPR4	CCG GTA TGG AAG ATG AAA AAG TC			
*phz*C	PHZJR1	CAG GGC CG(G/C)(A/G)(C/T)A TTT CTC GGT TCT	94°C for 2 min, 30 cycles of 94°C for 1 min, 67°C for 45 s, 72°C for 1 min, 72°C for 10 min	522	Mazurier *et al*. (2009)
	PHZJR2	GCG CGG GTC GCA CAG G CTT TTG TA			
*prn*C	PrnCf	CCA CAA GCC CGG CCA GGA GC	94°C for 2 min, 30 cycles of 94°C for 1 min, 58°C for 45 s, 72°C for 1 min, 72°C for 10 min	719	Mavrodi *et al.* (2001)
	PRrnCr	GAG AAG AGC GGG TCG ATG AAG CC			

3.2.10 Analysis of the fungal culture filtrates using NMR (nuclear magnetic resonance) spectroscopy

The NMR analysis was performed by Dr John Van Klink at the Plant and Food Research (Dunedin, New Zealand). The cell free fungal culture filtrates prepared in Section 3.2.7 were further analysed to check if any of the isolates produced polygodial or 9-deoxymuzigadial. The protocol from Perry *et al.* (1996b) was adapted for the analysis in this study. The filtrates were washed with 30 mL chloroform and evaporated on a rotary evaporator. The resulting dry extract was then taken up in Cadmium chloride ($CdCl_3$) which was used as the NMR solvent. The solvent was then subjected to NMR analysis and the resulting peaks of the filtrate were compared to the peaks of *P. colorata* dialdehydes polygodial, and 9-deoxymuzigadial.

3.2.11 Statistical analysis

To identify any differences in bioactivity against the test pathogens based on region of origin, Pearsons chi-square test was performed at $P<0.05$ using Minitab 17 (Lead Technologies, Australia).

3.3 Results

3.3.1 Culture collection of endophytic bacteria and fungi

A total of 350 endophytic bacteria and 200 fungi were recovered from the surface sterilized tissues of *P. colorata*. Most of the endophytic bacteria were isolated from the stem (57.1%, n=200), followed by roots (37.1%, n=130) and leaves (5.7%, n=20) (Appendix B.5). No bacteria grew on the plates on which the leaf imprints were taken and the wash water was plated demonstrating that the surface sterilization process was effective.

3.3.2 Activity of endophytic bacteria against phytopathogenic fungi:

Of the total endophytic bacteria (n=350) screened against the phytopathogenic fungi *N.luteum* ICMP 16678*, N. parvum* MM562*, I. liriodendri* WPA1C and *N. ditissima* ICMP 14417, 11 strains showed activity against all the phytopathogenic fungi tested, seven strains were active against at least two test pathogens (Fig. 3.3). The bioactivity was classified on the basis of the size of the zone of inhibition against the test pathogen, high

activity (inhibition zone > 3 mm), moderate activity (inhibition zone < 3 mm but ≥ 2 mm), low activity (inhibition zone < 2 mm but ≥ 1 mm) (Fig. 3.4) (Appendix B.5).

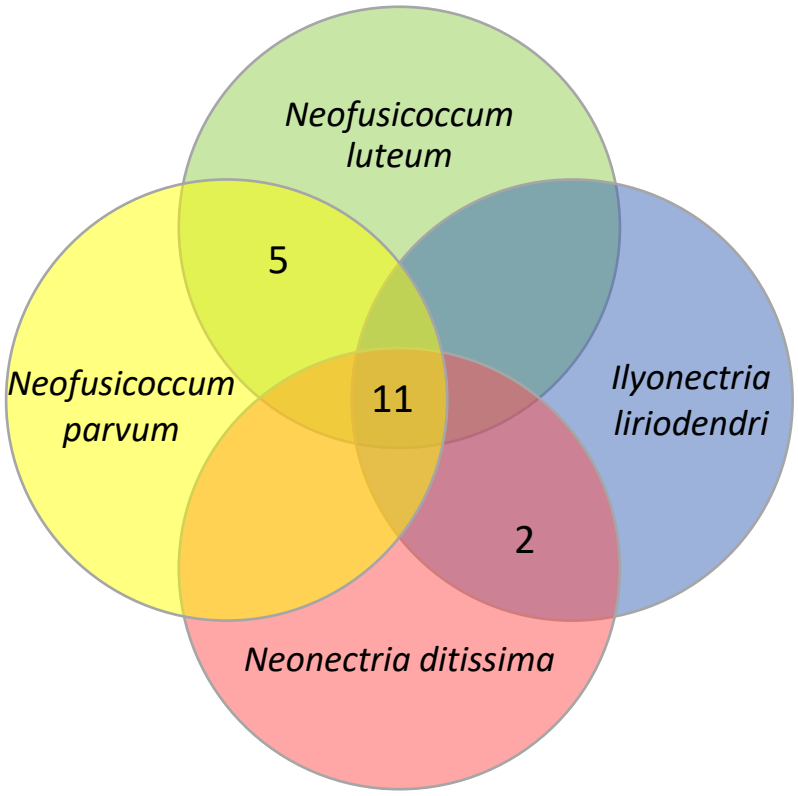


Figure 3.3: Venn diagram depicting the number of endophytes from *Pseudowintera colorata* active against the four phytopathogenic fungi tested.

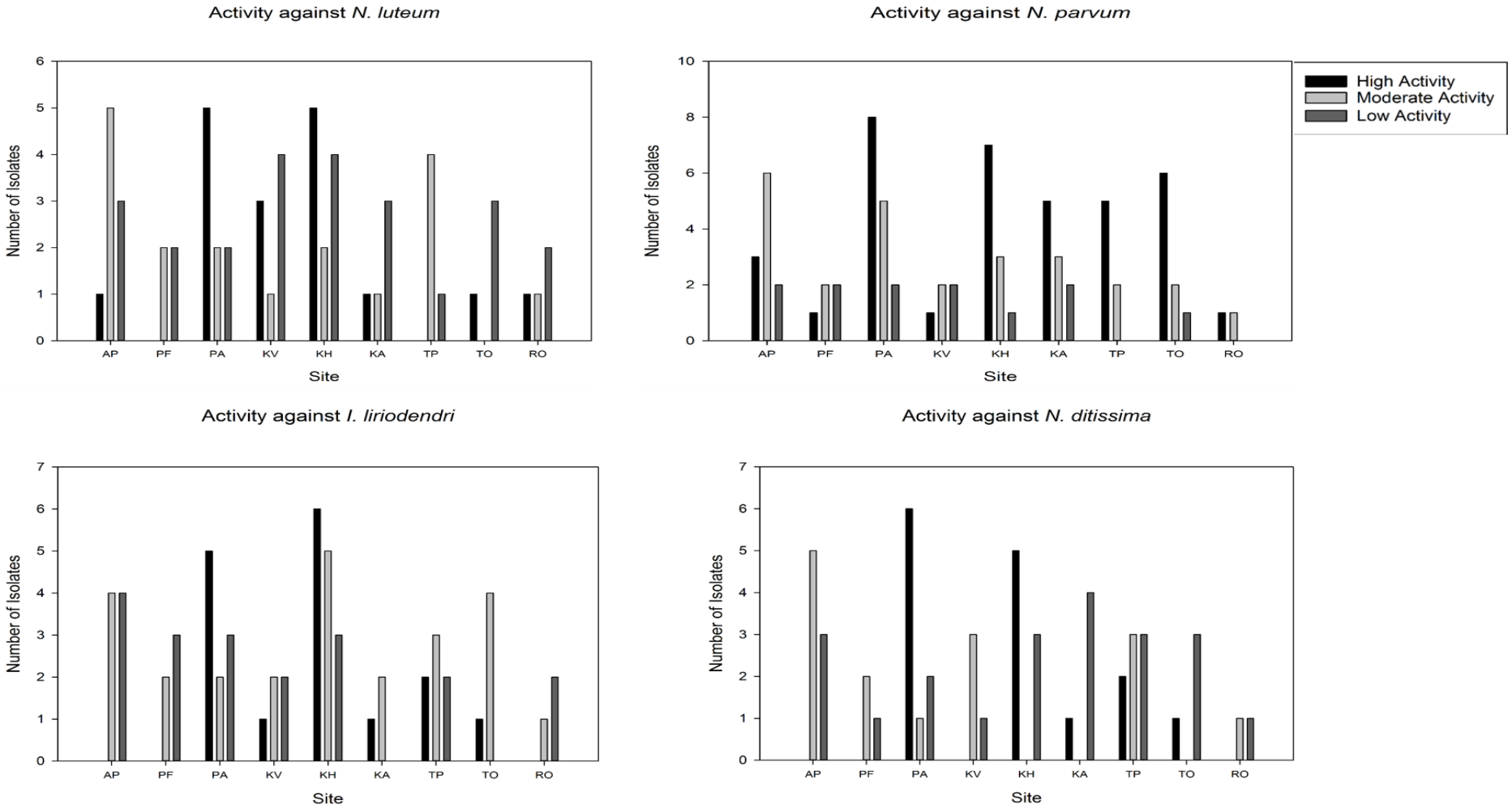


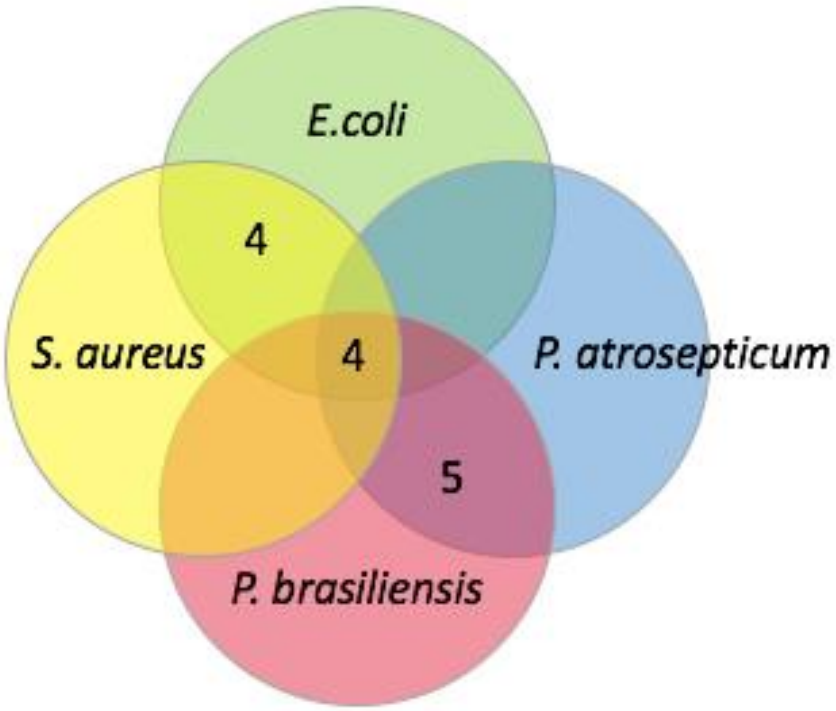
Figure 3.4: Bar graphs depicting the number of bacteria from each site showing activity against the phytopathogenic fungi *Neofusicoccum luteum, Neofusicoccum parvum, Ilyonectria liriodendri* and *Neonectria ditissima* (AP=Arthurs Pass, PF= Peel Forest, PA= Paringa Forest, KV=Kaituna Valley Reserve, KH= Kahurangi National Park, KA= Kaimanawa Forest Park, TP= Taihape Scenic Reserve, TO= Tongariro National Park, RO= Lake Rotopounamu.

Isolates with high activity were sequenced and identified as bacteria belonging to genera *Bacillus*, *Pseudomonas* and *Pantoea*.

3.3.3 Activity against phytopathogenic bacteria and opportunistic human pathogens

Of the isolates screened (n=350), strains *Bacillus* sp. KIP1SB1B, *Bacillus* sp. PRY2BBC1, *Pantoea* sp. AP1SA1, and *Bacillus* sp. PR1BC2U (Appendix B.5) were active against all the bacterial pathogens tested (Fig. 3.5 A). *Bacillus* sp. PR1BBb1 was active against both *Pectobacterium* species tested but had no activity against the opportunistic human pathogens (Fig. 3.5 B). Six strains were active against at least two test pathogens, five strains were active against one test pathogen. Only strain *Bacillus* sp. TP1LA1B showed moderate to low activity against *C. albicans*.

A



B

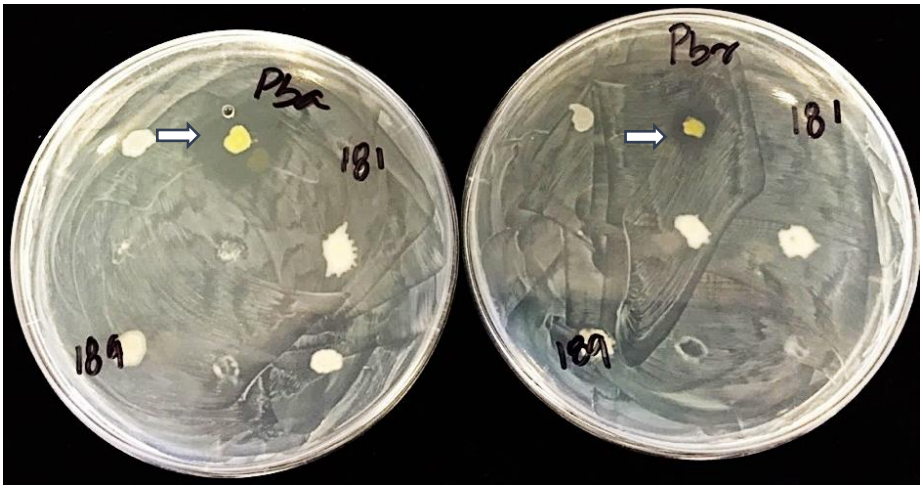


Figure 3.5: A) Venn diagram depicting the number of endophytes active against bacterial pathogens tested B) Activity of endophytes against *Pectobacterium atrosepticum* and *P. brasiliensis*; arrows indicating zones of clearance around the positive strains.

3.3.4 Secretion of siderophores

Out of the bacteria screened (n=350), 6.5% of isolates produced orange halos of size >17.5 mm; 6.9% isolates produced halos of size between 10-15 mm (Table 3.2, Appendix B.5). High activity was observed from isolates recovered from Taihape scenic reserve, Kaimanawa forest park and Lake Rotopounamu (Fig. 3.6). The isolates producing halos >17.5 mm also showed activity against phytopathogens.

Table 3.2 Percentage of isolates producing siderophores on Chrom-azurol S agar plates

Siderophore production	% of isolates
Zone >17.5 mm	6.5
Zone 10-15 mm	6.9
Zone < 5mm	20.6
No activity	66.0

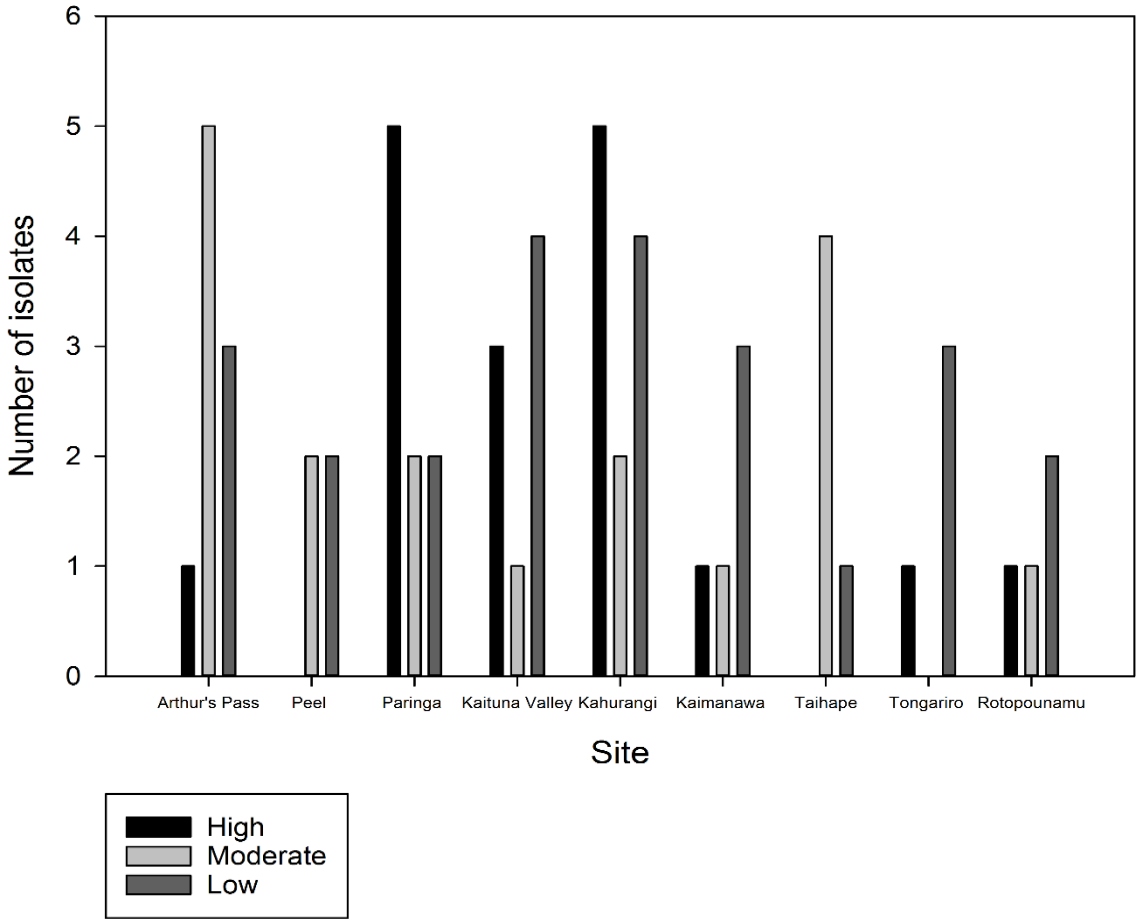


Figure 3.6: Number of endophytes recovered from *Pseudowintera colorata* from different New Zealand sites that produced siderophores on Chrom-azurol S agar plates. High activity indicates orange halos of size >17.5 mm and moderate activity halos of size between 10-15 mm.

3.3.5 Secretion vs induction assays of the bioactive compounds

Prior inoculation of the bioactive strains on the test plates showed greater inhibition of the test pathogens indicating that the bioactive metabolites were secreted without the presence of an antagonistic agent and that they accumulate over time (Fig. 3.7).

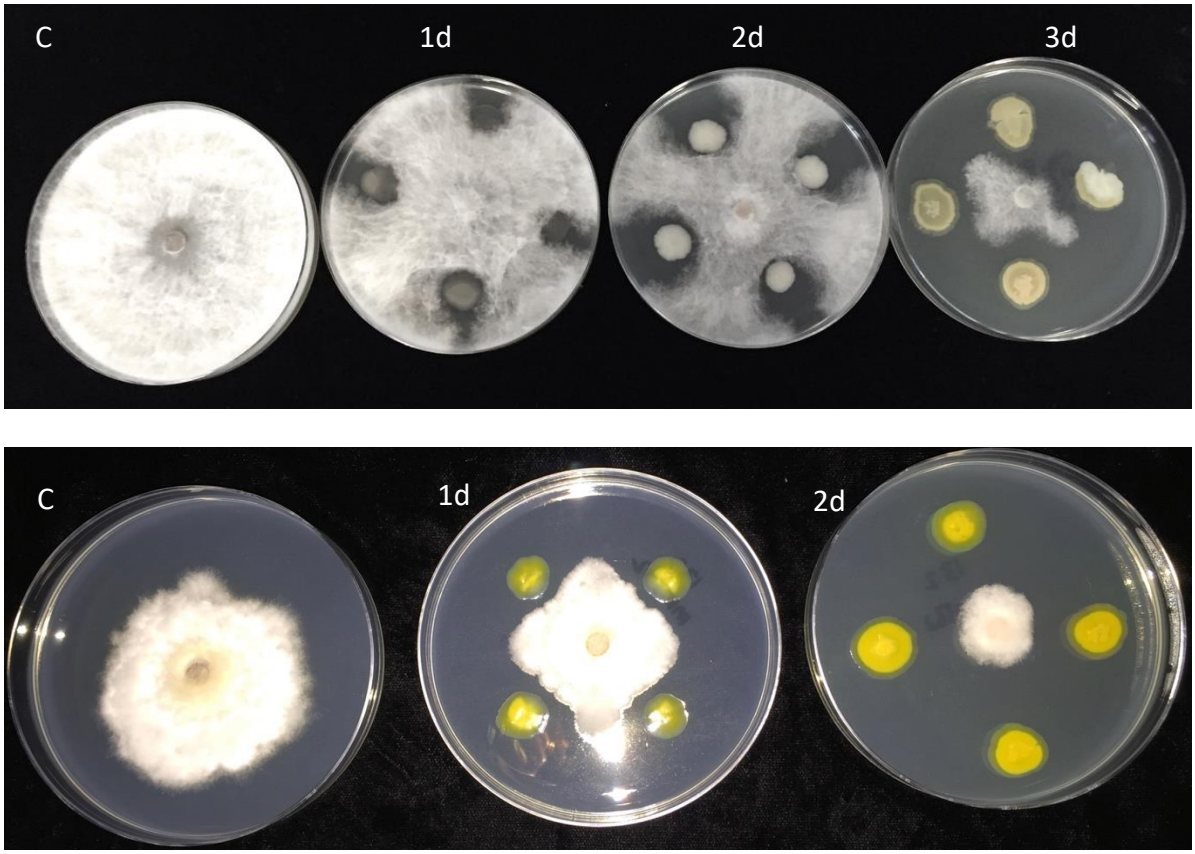


Figure 3.7: Activity of endophytes against *Neofusicoccum luteum* (Top) and *Neofusicoccum parvum* (Bottom) on plates showing the time of inoculation of bacteria (C= Control, 1 d = 1 day, 2 d = 2 days and 3 d = 3 days (*N. luteum* only) prior to inoculation).

3.3.6 Secretion of bioactive metabolites in liquid culture and activity against bacterial phytopathogens

Cell free supernatants of strains *Bacillus* sp. KIP1SB1B, *Pantoea* sp. AP1SA1 and *Bacillus* sp. PR1BC2U were active against all the bacterial pathogens tested. *Pseudomonas* sp. PR1BBb1 was active against both *Pectobacterium* species. *Pantoea* sp. AP1SA1 was active against *S. aureus* 297 and *E. coli* 916 (Fig. 3.8). Filtrate of *Bacillus* sp. TP1LA1B showed a small zone (≤2 mm) of clearance against *C. albicans*.

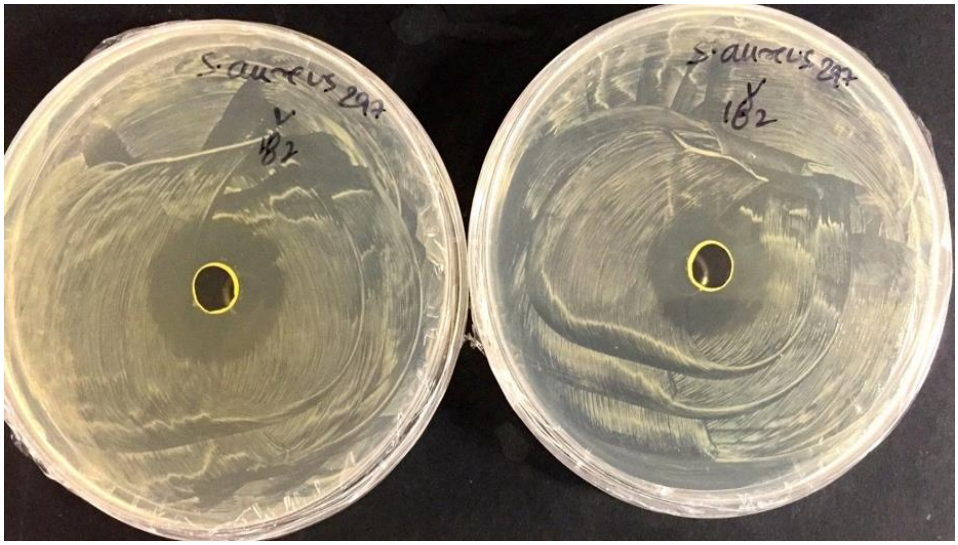


Figure 3.8: Activity of cell free supernatant against *Staphylococcus aureus*. Clearance zone around the well indicate positive activity.

3.3.7 Functional activity of endophytic fungi against *C. albicans*

To test for production of inhibitory metabolic compounds the test endophytic fungi were grown on cellulose membranes on the agar plate. These membrane with the fungal mycelia were removed prior to inoculation with *C. albicans*. Of the total endophytic fungi tested (n=50), metabolites produced by PRY2BA21 and P4BB2 completely inhibited *C. albicans* on the plates; PRY2BA2, P4LC2, P4LA3, and PR1BC2 produced clearance zones greater than 7 mm (Fig. 3.9).

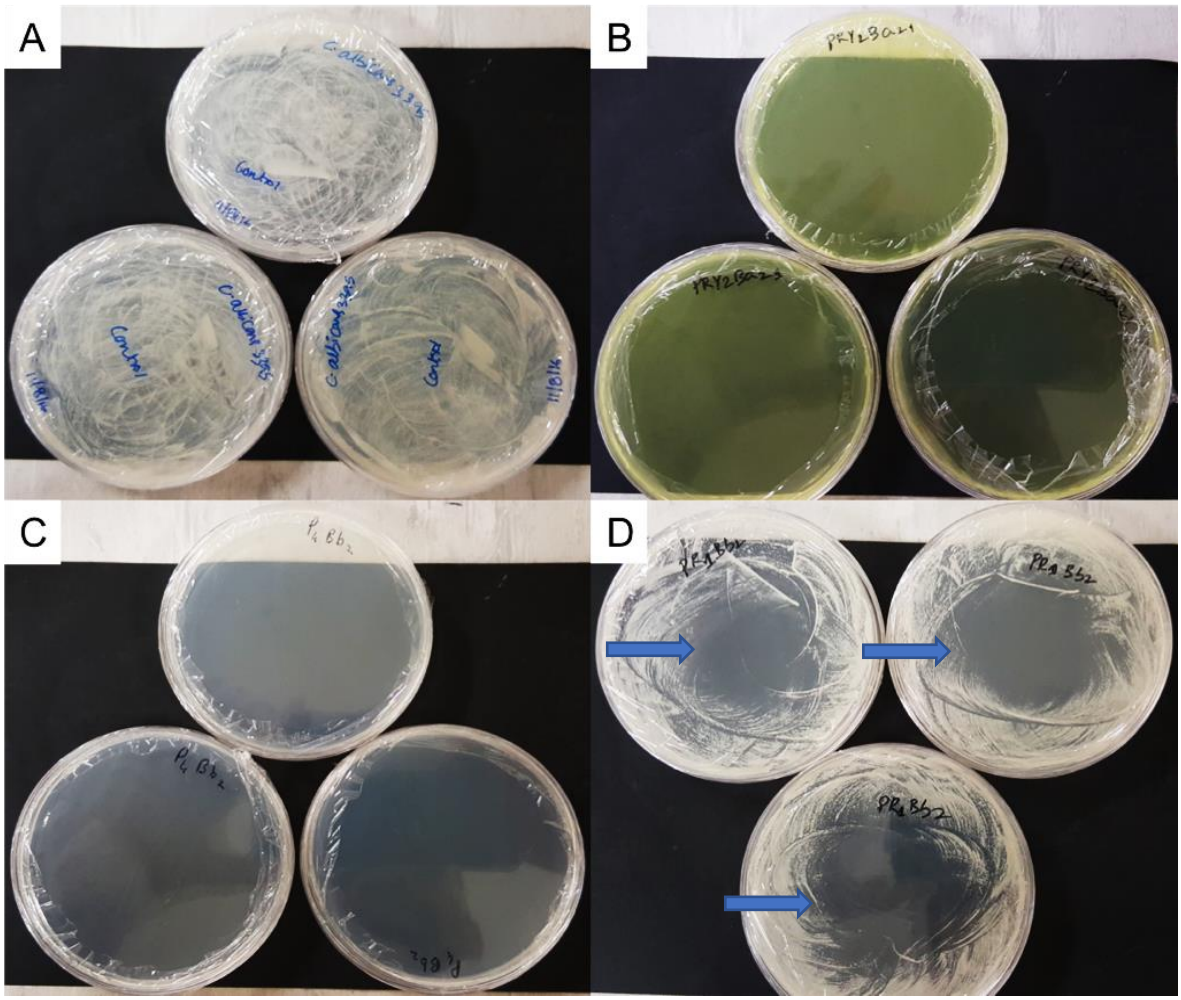


Figure 3.9: Activity of fungal endophyte metabolites against *Candida albicans* A) *C. albicans* control B) and C) plates with full clearance zones D) arrows indicating the clearance zones in the centre of the plate with *C. albicans.* The cellulose membranes on agar plates with the endophytic fungi were removed prior to inoculation with *C. albicans* on agar.

3.3.8 Effective concentration of metabolites against test pathogens

The soluble metabolites from *Metarhizium* sp. PR1SB1 and *Pezicula* sp. PRY2BA2 showed activity against *N. parvum* and *N. luteum* at both concentrations. However, the effect was predominant in the plates with 10% concentration (Fig.3.10).

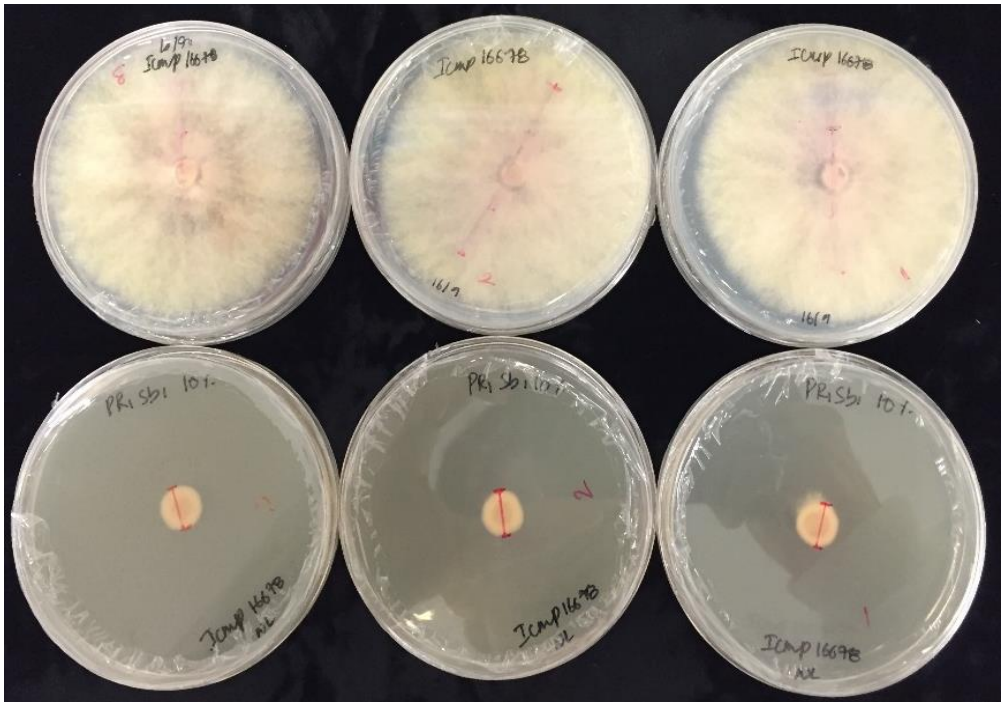


Figure 3.10: Effect of endophytic fungal filtrate on the growth of *Neofusicoccum luteum*; Top row: controls, Bottom row: agar incorporated with 10% filtrate after 3 days.

3.3.9 Identification of culturable bacteria and fungi

The bacterial and fungal endophytes showing activity against phytopathogens and opportunistic pathogens were sequenced and identified as members belonging to genus *Pseudomonas, Bacillus, Erwinia, Pantoea, Trichoderma, Pezicula, Metarhizium, Fusarium,* and *Chaetomium* (Table 3.3).

Table 3.3 Identity of endophytic bacteria and fungi that showed highest activity *in vitro* against a range of fungal and bacterial phytopathogens and human pathogens based on 16S rRNA and ITS 2 sequencing

	N. luteum	*N. parvum*	*I. liriodendri*	*N. ditissima*	*P. atrosepticum*	*P. brasiliensis*	*C. albicans*	*S. aureus*	*E. coli*
Bacillus sp. TP1LA1B	+++	++	++	++	++	-	+	+++	-
Bacillus sp. TP1LC1B	+++	++	++	++	-	-	-	+++	-
Bacillus sp. TOYPRB1R	+++	++	++	++	-	-	-	+++	-
Bacillus sp. KIP1SB1B	+++	++	++	++	++	++	-	+++	+++
Pseudomonas sp. KRP1BA1	+++	+++	++	++	-	-	-	++	-
Pantoea sp. AP1SA1	-	-	-	-	+++	+++	-	+++	+++
Pseudomonas sp. KRP1BC1	+++	+++	-	-	-	-	-	++	-
Pseudomonas sp. KRP1BB1	+++	+++	++	++	-	-	-	-	-
Pseudomonas sp. KRP1BA1	+++	+++	++	++	-	-	-	++	-
Erwinia sp. KVP1BC1	-	++	-	-	-	-	-	-	-
Pezicula sp. PRY2BA2	+++	+++	+++	+++	-	-	+++	+++	-
Metarhizium sp. PR1SB1	+++	+++	+++	+++	-	-	-	+++	-
Trichoderma sp. PRY2BA21	+++	+++	NT	NT	NT	NT	+++	NT	NT
Fusarium sp. P4LC2	NT	NT	NT	NT	NT	NT	+++	NT	NT
Xylaria sp. P4BB2	NT	NT	NT	NT	NT	NT	+++	NT	NT
Chaetomium sp. PR1BC2	NT	NT	NT	NT	NT	NT	+++	NT	NT
Xylaria sp. P4LA3	NT	NT	NT	NT	NT	NT	+++	NT	NT

+++ High activity, ++ moderate activity, + low activity, - no activity, NT- Not tested

3.3.10 Detection of antibiotic producing genes from bacteria

PCR amplification revealed that of the bacterial isolates tested (n=9), genes for the production of phenazine were detected in only one isolate (*Pseudomonas* sp.KRP1BB1). 2,4-DAPG and pyrollnitrin producing genes were not detected in any of the isolates tested.

3.3.11 Analysis of endophytic metabolites using NMR

Analysis of the NMR spectra revealed that none of the filtrates had peaks that corresponded to the signals of dialdehydes (polygodial and 9-deoxymuzigadial) in *P. colorata* indicating that the cell free filtrates did not contain these compounds.

3.4 Discussion

This study is the first to describe the isolation and bioactive potential of culturable bacterial and fungal endophytes from *P. colorata*. All the *P. colorata* tissues selected for isolation of endophytic bacteria and fungi were free from any visible symptoms of disease and herbivory. The bioactive potential of the endophytic bacteria and fungi against phytopathogenic bacteria and fungi, and human pathogenic bacteria and yeast was investigated using *in vitro* assays. Strains which demonstrated high activity against phytopathogens, and secreted siderophores were identified using 16S rRNA sequencing. These selected endophytes may have potential effect on *P. colorata* growth, which will be assessed in Chapter 5.

For the assays set up in this study, the phytopathogenic fungi *Neofusicoccum parvum*, *N. luteum, Neonectria ditissima and Ilyonectria liriodendri* and phytopathogenic bacteria *Pectobacterium atrosepticum* and *P. brasiliensis* were selected on the basis that these pathogens are aggressive pathogens in New Zealand and globally with little success achieved though chemical fungicides and bactericidal agents (Gnanamanickam and Charkowski, 2006; Czajkowski *et al*., 2011, Augustí-Brisach and Armengol, 2013). The diseases caused by these pathogens range from tuber rot, trunk disease, stem cankers to root rot and eventually lead to death of the plant.

In this study, all the tissues of *P. colorata* sampled (roots, stems and leaves) hosted at least one culturable endophyte. These results support those of Strobel and Daisy, (2003) that all the individual plants on earth are colonized by one or more endophyte. The number of endophytes isolated from same tissue type from different sites was different. These results were similar to Park *et al.* (2012) in ginseng plants, where they isolated more endophytic fungi

from 4 year old plants compared to 3 year old plants. As the *P. colorata* plants were sampled from DOC preserved nature parks, the age of the plants sampled was only estimated based on the height and girth of the stem. In order to understand if maturity is a key reason for the variation in recovery of culturable endophytes from the same tissue type, plants of known different age groups will have to be sampled in future studies.

Although in several comparable studies, roots were reported to host a higher number of endophytes (bacteria and fungi) compared to stems and leaves (Mano *et al*., 2007; Verma *et al*., 2013; Jin *et al*., 2014; Wicaksono *et al*., 2016), in this study, the number of bacterial and fungal endophytes recovered from stems was higher than the roots and leaves. These results were similar to Verma *et al.* (2013) studying the endophytic fungi in an Indian medicinal plant *Madhuca indica*, where they observed a greater diversity of fungi in stems. The possible explanations being: 1) differences in the growing conditions at the site, 2) differences in the age of *P. colorata* plants sampled, 3) reaction to surface sterilization agents (Mercado-Blanco and Lugtenberg, 2014) or 4) differences in the spore abundance of dominant species colonizing the stem tissues (Verma *et al.*, 2013). In addition, roots and leaves of *P. colorata* were quite fleshy and supple and likely imbibed more of the sterilizing agents compared to the stem tissues. Verma *et al.* (2011) reported that the addition of alcohol during surface sterilization could enhance the wetting, penetrating and killing properties of NaOCl, which could have killed some endophytes in this study. Comparison of different sterilisation procedures could further optimise the sterilisation process used here.

Leaves of *P. colorata* yielded the lowest number of culturable bacterial endophytes (5.7%, n=20). The low number of culturable endophytes from the leaves of *P. colorata* could be because they contain the sesquiterpene dialdehyde polygodial which is known to have a very strong activity against bacteria and fungi (Kubo *et al.,* 2001; Kubo *et al.,* 2005). Cooney *et al.* (2012) reported that the leaves of *P. colorata* contain spherical oil vesicles called idioblasts, which were likely the sites of polygodial biosynthesis and storage. During the recovery of endophytes, after dissecting the leaf, the endophytic bacteria and fungi may have been killed due to the direct contact with polygodial from the idioblasts.

The endophytic bacteria (n=350) and fungi (n=200) were selected based on their morphology during initial isolation. Similar studies by Liu *et al.* (2016) and Wicaksono *et al*. (2016) on the medicinal plants *Ferula songorica* (Chinese medicinal plant) and *L. scoparium* tissues,

respectively, isolated 170 and 192 culturable endophytic bacteria. The endophytic fungi were grouped based on their morphology and characteristics. From these groups, a representative set (n=50) was selected for screening studies. Due to this selection process, other fungi, which may have similar morphology but belong to a different genus may have been excluded. In addition, some of the slow growing bacterial and fungal endophytes may not have been recovered due to the fast growing and sporulating isolates overgrowing on the plate (Rosenblueth and Martinez Romero, 2006; Verma *et al.,* 2011). Preference for the recovery media may also have affected the number of endophytes recovered from the tissues (Verma *et al.,* 2011). The media, R2A (Difco) and SNA (SIFIN), used in this study have been used routinely in the recovery of endophytes (Kusari *et al*., 2012; Nissinen *et al*., 2012; Yi Shen and Fulthorpe, 2015). Also, the number of endophytes recovered from tissues was also likely to be dependent on the relative abundance of culturable and unculturable taxa. Due to years of co-evolution some endophytes may not be able to survive without the host tissue, be sequestered from the toxic compounds in the host tissue or have entered a viable but not culturable (VBNC) state during processing as a mechanism of survival (Mercado-Blanco and Lugtenberg, 2014).

Several studies have shown that endophytes do confer unique properties to the host (Li *et al.,* 2016b). This study revealed that the endophytic bacteria and fungi inhabiting *P. colorata* have antimicrobial properties. Several of the bacterial and fungal endophytes of *P. colorata* demonstrated antagonistic activities against phytopathogenic bacteria, phytopathogenic fungi and opportunistic human pathogens and produced siderophores.

In total, 34% (n= 119) of the culturable bacterial endophytes in this study demonstrated the ability to produce siderophores to chelate iron from CAS agar. These results were similar to the findings of Wicaksono *et al.* (2016) on *L. scoparium* endophytic bacteria, which demonstrated a high frequency of siderophores producing members. In this study, several bacterial endophytes (n=21) producing siderophores and also showing antagonistic activity against phytopathogenic bacteria and fungi were recovered from *P. colorata*. From the total endophytic bacteria tested (n=350), 9.14% (n=32), 13.1% (n=46), 9.14 (n=32), 10.2% (n=36) bacterial endophytes showed antagonistic activity against *N. luteum, N. parvum, I. liriodendri* and *N. ditissima,* respectively. Taurian *et al*. (2010) observed that 10% of the culturable bacteria (n=11) from peanut showed antagonistic activity against *Sclerotinia minor* and *S.*

sclerotiorum. Similar results were demonstrated by Wicaksono *et al*. (2016) where they found that the *L. scoparium* endophytic bacteria which solubilized phosphate and produced siderophores were also active against *Neofusicoccum* sp., *Ilyonectria* sp. and *Pseudomonas syringae* pv. *actinidae* (Psa) *in vitro*.

The culturable endophytic bacteria and fungi with the highest inhibitory activity against test pathogens (phytopathogenic bacteria, fungi and opportunistic human pathogens) were sequenced and were identified as members belonging to the genus *Bacillus*, *Pseudomonas*, *Pantoea*, *Trichoderma, Chaetomium, Fusarium, Pezicula, Metarhizium* and *Xylaria*. Bacteria belonging to the genus *Pseudomonas* are one of the most abundant genera and known for their bioactive properties (production of antibiotic like compounds, antagonistic activity against phytopathogens, production of siderophores) in several medicinal plants like *L. scoparium* (Wicaksono *et al.,* 2016), *Lavandula angustifolia* (Emiliani *et al.*, 2014), *Aloe barbadensis* (Gupta *et al.*, 2012) and non-medicinal plants like *sphagnum* moss (Shcherbakov *et al.*, 2013). *Pseudomonas* sp. are known to produce several different antibiotics such as 2, 4- diacetylphloroglucinol, pyrollnitrin, pyoluteorin and phenazine (McSpadden *et al*., 2001; de Souza and Raaijmakers, 2003; Mazurier *et al*., 2009). Of the bioactive *Pseudomonas* strains in this study only one was shown to produce an antibiotic (phenazine), indicating that the antagonistic potential of the strains is likely to be due to other compounds. From the antagonism assays, one *Bacillus* sp. was active against *C. albicans* and all phytopathogenic fungi tested. Arguelles-Arias *et al*. (2009) reported that genus *Bacillus* is known to produce a variety of compounds including the antibiotic zwittermycin A, which has activity against several phytopathogens like *Pythium* sp. and *Phytophthora* sp. In addition, the *Bacillus* sp. in this study, produced siderophores.

Endophytic fungi have been identified for their potential in the synthesis of a wide variety of biologically active compounds (Aly *et al.,* 2011; Gond *et al*., 2012; Bezerra *et al.,* 2015) including the same (or similar) compounds for which the host plant is recognised. For example, Stierle *et al*. (1993) identified that the endophytic fungi *Taxomyces andreanae* was capable of producing the anticancer drug, Taxol™ (Paclitaxel) similar to its host Pacific yew tree, *Taxus brevifolia* (Wani *et al.,* 1971). Similarly, the anticancer drug campothecin, the anticancer drug lead compound podophyllotoxin and a natural insecticide azadirachtin are co-produced by the plant and their associated endophytes (Puri *et al.,* 2005; Puri *et al.,* 2006;

Kusari *et al.,* 2012). The bioactive potential of randomly selected representative (n=50) endophytic fungi isolated from *P. colorata* was tested against *C. albicans* which is the target of polygodial. Of the strains tested 26% (n=13) of isolates showed activity against *C. albicans*, with 14% (n=7) of isolates showing high activity (zone of inhibition> 10mm). Similar results were found by Bezerra *et al.* (2015), where 34.3% of the endophytic fungi isolates from the Brazilian medicinal plant *Bauhinia forficata* showed activity against *S. aureus, E. coli*, and *Streptococcus pyogenes*. *Bauhinia forficata* species have been reported to have activity against *Aspergillus*, *Cladosporium, Cryptococcus, Candida* and *Salmonella* (da Silva and Cechinel-Filho, 2002).

Sequencing the ITS2 region revealed that the bioactive fungi belonged to the genera *Trichoderma, Pezicula, Fusarium, Metarhizium, Chaetomium* and *Xylaria*. Cui *et al.* (2011) found that the endophytic fungi belonging to the genus *Fusarium* isolated from the medicinal plant *Aquilaria sinensis* had strong activity against *S. aureus* and *Bacillus subtilis*. Vicente *et al.* (2001) found that ergokonin A, produced by *Trichoderma* is active against *C. albicans*. In this study, the fungi identified as belonging to the genus *Pezicula* showed strong activity against all the phytopathogenic fungi tested, the opportunistic human pathogen *S. aureus* strain 297 and the *C. albicans* strain 3395. Strobel *et al*. (1999) and Noble et al. (1991) reported that certain species of *Pezicula* produce one or more lipopeptide antimycotics known as pneumocandins and several other compounds such as (R)- Mellein Echinocandin A, which is recognized for its activity against *C. albicans* along with other bacteria (*S. aureus*) and fungi (*Ustilago violacea*). Analysis of the fungal culture filtrates using NMR revealed no peaks in the dialdehydes region, indicating that the filtrates did not contain polygodial and 9-deoxymuzigadial. Though the culture filtrates did not have any peaks corresponding to the dialdehydes, the ability of fungal mycelia to sequester these compounds cannot be ruled out and needs to be examined in future studies.

In conclusion, *P. colorata* contains endophytic bacteria and fungi with bioactive properties, which have potential as antimicrobial agents. There were also several with activity against *C. albicans*. These results support the findings of Chapter 2 that used molecular methods to show that *P. colorata* harboured a wide diversity of endophytic bacteria and fungi. However, the effect of these endophytes on the host plant and their role *in planta* have not been studied previously and will need additional studies to understand their functions better.

Chapter 4

Diversity, structure and function of Actinobacteria in *Pseudowintera colorata*

4.1 Introduction

Phylum Actinobacteria is one the largest phyla among bacteria and represents bacteria that are gram-positive, aerobic spore forming, have a high G+C content in their DNA and recognized for their bioactive metabolites (Stackebrandt *et al.,* 1997; Ludwig and Klenk, 2005; Golinska and Dahm, 2011). They play an important role in decomposition of complex organic matter from plants, animals and in recycling nutrients in the form of humus (Sharma, 2014). Studies have shown that Actinobacteria can form close associations with plants and colonize their tissues (Qin *et al*., 2012). Of approximately 33,000 known microbial bioactive metabolites, those derived from Actinobacteria account for 12,000 (about one third), with *Streptomyces* alone known to produce 7600 compounds (Berdy, 2012). Studies by Ryan *et al*. (2008) and Bascom-Slack *et al*. (2009) have revealed that many plants are rich in endophytic Actinobacteria which produce compounds with diverse functions. Although there have been published studies that have examined the effect and impact of abiotic factors and inoculants on Actinobacteria communities in New Zealand soils (Stark *et al*., 2007; Shi et al., 2011), there are no published studies about Actinobacteria as endophytes of native plants in New Zealand. This is the first study to identify the culturable and non-culturable endophytic Actinobacteria in *P. colorata*.

Studies by research groups have shown that endophytic Actinobacteria can be used as biocontrol agents and, in addition to protecting the host plant, they can enhance growth and tolerance of adverse conditions (Igarashi *et al*., 2002; Hasegawa *et al*., 2006; Cao *et al*., 2005). In addition to turning over organic matter in the soil, studies have shown that endophytic Actinobacteria can colonize plants, promote plant growth through assimilation of iron and other nutrients (Tokala *et al*., 2002; Coombs and Franco, 2003b) and protect plants against phytopathogens by producing antibiotics *in situ* (Cao *et al*., 2004). As a result of their long term association with the host plant, endophytic Actinobacteria may also be involved in the metabolic pathways of the plant and, in some instances, they have gained host genetic information allowing them to produce compounds similar to the host (Kumar *et al.,* 2013; Rai *et al.,* 2014; Golinska *et al.,* 2015). Pujiyanto *et al*. (2012), working on the Indonesian

antidiabetic medicinal plant *Tinospora crispa,* isolated an endophytic *Streptomyces* sp. which was capable of secreting twice the amount of alpha glucosidase inhibitor (antidiabetic) as the host plant. Apart from the production of beneficial compounds endophytic Actinobacteria can promote plant growth through production of siderophores (Compant *et al*., 2005a; Nimnoi *et al*., 2010). Siderophores are low molecular weight compounds (<1000 Da) which have a high iron affinity (Neilands, 1984; Neilands, 1995). Researchers have reported that genera of Actinobacteria like *Streptomyces*, *Nocardia* and *Rhodococcus* produce several types of siderophores such as desferrioxamine, enterobactin, coelichelin, griseobactin and heterobactin (Challis *et al*., 2000; Fiedler *et al*., 2001; Mukai *et al*., 2009; Patzer and Braun 2010).

The main objectives of this chapter are to:

1) Identify the Actinobacteria communities in *P. colorata* using culture independent methods
2) Isolate and culture endophytic Actinobacteria from tissues of *P. colorata.*
3) Identify isolates with bioactive potential using *in-vitro* functionality assays.
4) Study the effects of bioactive strains *in-vivo* on the growth of *P. colorata.*

4.2 Materials and Methods

4.2.1 Sampling locations

Sampling locations of *P. colorata* were as described in Section 2.2.1

4.2.2 Plant sampling

Pseudowintera colorata tissues used for this study were sampled as described in Section 2.2.2

4.2.3 Sample processing

Tissues of *P. colorata* were processed as described in Section 2.2.3

4.2.4 Isolation and preservation of Actinobacteria

Actinobacteria are very slow growing bacteria and to increase the chances of isolating different strains, the surface sterilized tissues (leaves, stems and roots), were plated onto Bennett's agar (Appendix C.1) and Starch Casein Agar (Appendix C.2) which are Actinobacteria selective media. Both the agar were amended with the antifungal agents Nystatin and

cycloheximide (50 μg/mL) (Passari *et al*., 2015) to prevent the growth of fungi. The plates were incubated at 25°C in the dark and observed daily for Actinobacteria. Emerging colonies with morphology typical of Actinobacteria, being powdery or elevated with margins pulling the agar were sub-cultured individually onto Bennett's agar plates. Colonies were subcultured at least twice to ensure purity. The pure cultures were then streaked onto sterile agar slants and incubated for 7-10 d at 25°C in the dark. Once the Actinobacteria colonies covered the agar surface, the slants were layered with 100% sterile glycerol and stored at -20°C.

4.2.5 Diversity analysis of actinobacterial communities in *P. colorata* using culture dependent and independent techniques

The diversity of Actinobacteria inhabiting *P. colorata* was analysed by both culture dependent techniques (isolation on to agar, functionality and 16S rRNA sequencing) and culture independent techniques (DGGE, NGS) as described in Sections 3.2.4, 2.2.5.1, 2.2.6 and 2.2.7.

4.2.5.1 Identification of the culturable and unculturable endophytic Actinobacteria

The endophytic Actinobacteria were identified by sequencing the 16S rRNA gene. The DNA for each strain was extracted using the PureGene kit (Qiagen) as per the manufacturer's instructions. Using the primer pair F243 (5'- GGA TGA GCC CGC GGC CTA -3') and R1494 (5'-TAC GGC TAC CTT GTT ACG AC -3') (Stark *et al.,* 2007; Nimnoi *et al.,* 2010), the 16S rRNA region was amplified. The PCR were carried out in an Applied Biosystems Proflex PCR system in a total volume of 25 μL containing 1 μL of template DNA, 2.5 μL buffer, 200 μM dNTPs, 1 U *Taq* DNA polymerase, 1 μL of each primer (10 μM) and 18.75 μL water. Positive (*Streptomyces* sp. isolate 151, isolated in this study) and negative controls were run for each PCR. The PCR conditions and visualization of the PCR products were as described in Section 3.2.8.

To identify the unculturable endophytic Actinobacteria, bands from the DGGE gels produced in Section 2.2.5.1 were excised using a sterile scalpel (Appendix C.3). The scalpel was sterilized using 96% ethanol and flaming after cutting every band. The bands were gently crushed using the scalpel edge and suspended in separate tubes containing 20 μL sterile PCR grade water and incubated overnight at 4°C in a fridge. The water from the tubes containing the DGGE bands was used as template for the PCR. PCR was performed using the primer pair F341-GC and R534 (Section 2.2.5.1) in a total volume of 25 μL containing 1 μL of template DNA, 2.5 μL buffer, 200 μM dNTPs, 1 U *Taq* DNA polymerase, 1 μL of each primer (10 μM) and 18.75 μL

water. *Streptomyces* sp. 151 was used as positive control and a negative control to which 1 μL of PCR water was added instead of DNA were run for each PCR. The PCR products were then loaded onto 1% agarose, separated by electrophoresis as described in Section 2.2.5 and visualized under UV light. The amplicons were sequenced directly at the Lincoln University Sequencing Facility. The sequences obtained were trimmed using DNAMAN v4 (Lynnon Biosoft, Canada) to remove ambiguous regions. The sequences were then compared against those of known origin using NCBI BLAST (basic local search alignment tool) and the GenBank database (www.ncbi.nlm.nih.gov). All the sequences were aligned using MUSCLE and the distance matrices and phylogenetic trees were calculated by maximum likelihood algorithms with 1000 bootstrap replication using MEGA 6 software (Molecular Evolutionary Genetic Analysis, Tamura *et al*., 2013).

4.2.6 Functional properties of endophytic Actinobacteria isolated from *P. colorata*

4.2.6.1 Activity against phytopathogenic fungi

The phytopathogenic fungi (*Neonectria ditissima*, *Neofusicoccum luteum*, *N. parvum* and *Ilyonectria liriodendri*) used in this study are as described in Section 3.2.5.1.

Dual culture assay

The assay was carried out on Waksman agar (WA, Appendix B.2) plates. Actinobacteria strains were revived from cold storage (-20°C) by streaking onto Waksman agar and the plates were incubated at 25°C for 10-15 d in the dark.

After 10 – 15 d, 6 mm discs of each Actinobacteria strain were excised and each placed 1 cm from the edge on a fresh Waksman agar plate. For Actinobacteria isolates that did not produce confluent growth pattern, the cultures were streaked 1 cm from the edge of the plate. The plates were incubated at 25°C for 7-10 d. After 7-10 d, a 6 mm discs of the fungal phytopathogen (*N. ditissima, N. parvum, N. luteum* and *I. liriodendri*) were placed 5 cm from the Actinobacteria colony in separate plates and the plates incubated again at 25°C in a 12 h light/12 h dark cycle for 3-7 d. The size of the zone of clearance around the Actinobacteria colony was recorded and compared to an uninoculated fungal control. The bioactivity was classified on the basis of the zone of inhibition against the test pathogen, high activity

(inhibition zone > 5 mm), moderate activity (inhibition zone < 5 mm but >/= 2 mm) and low activity (inhibition zone < 2 mm but > 1 mm).

4.2.6.2 Activity against phytopathogenic bacteria and opportunistic human pathogenic bacteria and yeasts

The phytopathogenic bacteria (*Pectobacterium atrosepticum* and *P. brasiliensis*) and opportunistic human pathogens (*Staphylococcus aureus* 297, *Escherichia coli* 916 and *Candida albicans* 3395) used in this study are as described in Sections 3.2.5.2 and 3.2.5.3.

Dual culture assay

Using a sterile cork borer, a 6 mm disc of each of the Actinobacteria strains were inoculated in the centre of WA plates and incubated as described in Section 4.2.6.1. After 7-10 d, using a sterile loop a single colony of the test pathogen was streaked from the centre of the plate towards the edge of the plate away from the Actinobacteria colony and the plates were sealed and incubated at 25°C for 24 h in the dark. Uninoculated control plates containing only the test pathogens were also set up. A clearance zone $\geq$ 1mm in radius around the Actinobacteria colony was recorded as positive.

4.2.6.3 Effect of secreted metabolites on test pathogens

A) Against phytopathogenic fungi

To test the effect of secreted metabolites on the test pathogens, two 6 mm plugs of the strains identified as showing activity from the dual culture assays (Section 4.2.6.1)were grown in flasks containing 150 mL of Waksman broth for 7-10 d in shaking incubator (Labnet 211DS) set at 25°C and 150 rpm in the dark. After 7-10 d the flasks were harvested and the cultures were centrifuged at 20,000 X *g* for 15 min to pellet the cells. The supernatant was filtered through a 0.22-micron pore size filter. The filtered supernatant was then incorporated into autoclaved Waksman agar cooled to 50°C to produce 1% and 10% (v/v) filtrate incorporated agar. Each filtrate incorporated agar was tested against the four fungal pathogens listed in Section 4.2.6.1 and replicated thrice. Controls were produced by growing each of the fungal pathogens alone on unamended Waksman agar and this was also replicated thrice. Plates were incubated at 20°C in 12 h light/12 h dark cycle. After incubation the amended agar plates

were compared with control plates and the colony diameter of the fungi on the plates was recorded.

B) Against bacterial phytopathogens and human pathogens

The test pathogens were grown overnight in nutrient broth incubated at 25°C. After 24 h, 100 μL of each of the overnight cultures was spread onto a separate Waksman agar plate using a sterile spreader. Using a sterile 6 mm cork borer, a well was made in the middle of the plate. To this well, 100 μL of the cell free supernatant separated as per Section 4.2.6.3 (A) was added. The plates were prepared in triplicate. For the control plates, the wells were inoculated with sterile nutrient broth. The plates were sealed and incubated at 25°C for 24 hours in the dark. The presence of a clear zone ≥ 0.5 mm around the well was noted as positive and the results were recorded in comparison to a control plate.

4.2.6.4 Secretion of siderophores

The ability of the Actinobacteria to secrete siderophores was tested on Chrom-azurol S agar (CAS Appendix B.3). Using a cork borer, 6 mm plugs of the Actinobacteria strains were inoculated face down in the centre of the CAS agar and the plates were incubated at 25°C in the dark. The plates were prepared in triplicate. The plates were observed daily for 7-10 d. Positive results were indicated by the presence of an orange halo around the Actinobacteria colony. The halo radius was measured using digital callipers.

4.2.6.5 Phosphate solubilisation

The ability of endophytic Actinobacteria to solubilize phosphate was tested on tricalcium phosphate (TCP, Appendix C.4) agar. The Actinobacteria cultures were revived from -20°C onto WA and incubated at 25°C for 7-10 d in the dark. After 7-10 d, using a sterile cork borer, a 6 mm plug was transferred and placed face down onto the centre of TCP agar plates and the plates were sealed and incubated at 25°C in the dark. The plates were prepared in triplicate. Three control plates were inoculated with a known phosphate solubilising *Bacillus* sp. provided by Kritarth Seth (Lincoln University, Plant Pathology Group). Positive results were indicated by the presence of a clear zone ≥ 0.5 mm around the colony. The clearance zone was measured from edge of the colony in two directions using a digital calliper.

4.3 Results

4.3.1 Identity of the culturable and unculturable endophytic Actinobacteria from the tissues of *P. colorata*

A total of nine endophytic Actinobacteria were cultured from the surface sterilized tissues (5 from stems and 4 from roots) of *P. colorata*. PCR of the 16S rRNA subunit using the primers F243 and R1494 yielded products approximately 1,500 bp long. Sequencing of these PCR products revealed that the strains belonged to the genera *Streptomyces* (n=3), *Nocardia* (n=1), *Micromonospora* (n=3), *Microlunatus* (n=1) and *Nakamurella* (n=1) (Fig. 4.1, Table 4.1). PCR of excised bands from DGGE gels using F341GC and R534 yielded PCR products approximately 150-180 bp long. Of 20 bands selected for PCR and sequencing from the DGGE gels, only 10 bands were successfully sequenced. Sequencing of the PCR product from the DGGE bands identified that bands 1L12B, 1L1B, 1L2A were uncultured bacteria. Bands L19A and 1L4B were identified as a strain of *Angustibacter peucedani* and a *Streptomyces* sp., respectively (Fig. 4.2, Table 4.1). Bands 2L2B, 2L7B, 1L4A, 1L5A and 1L5B were identified as chloroplast (Appendix C.5).

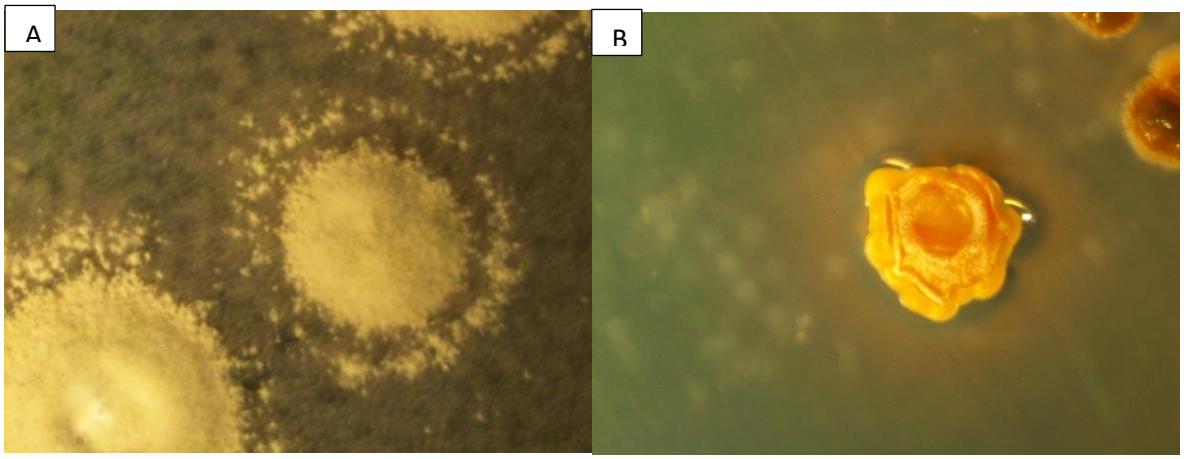


Figure 4.1: Colony morphology of endophytic Actinobacteria colonies recovered from surface sterilized tissues of *P. colorata* identified using 16S rRNA sequencing as A) *Streptomyces* sp. UKCW/B, B) *Nocardia* sp. TP1BA1B.

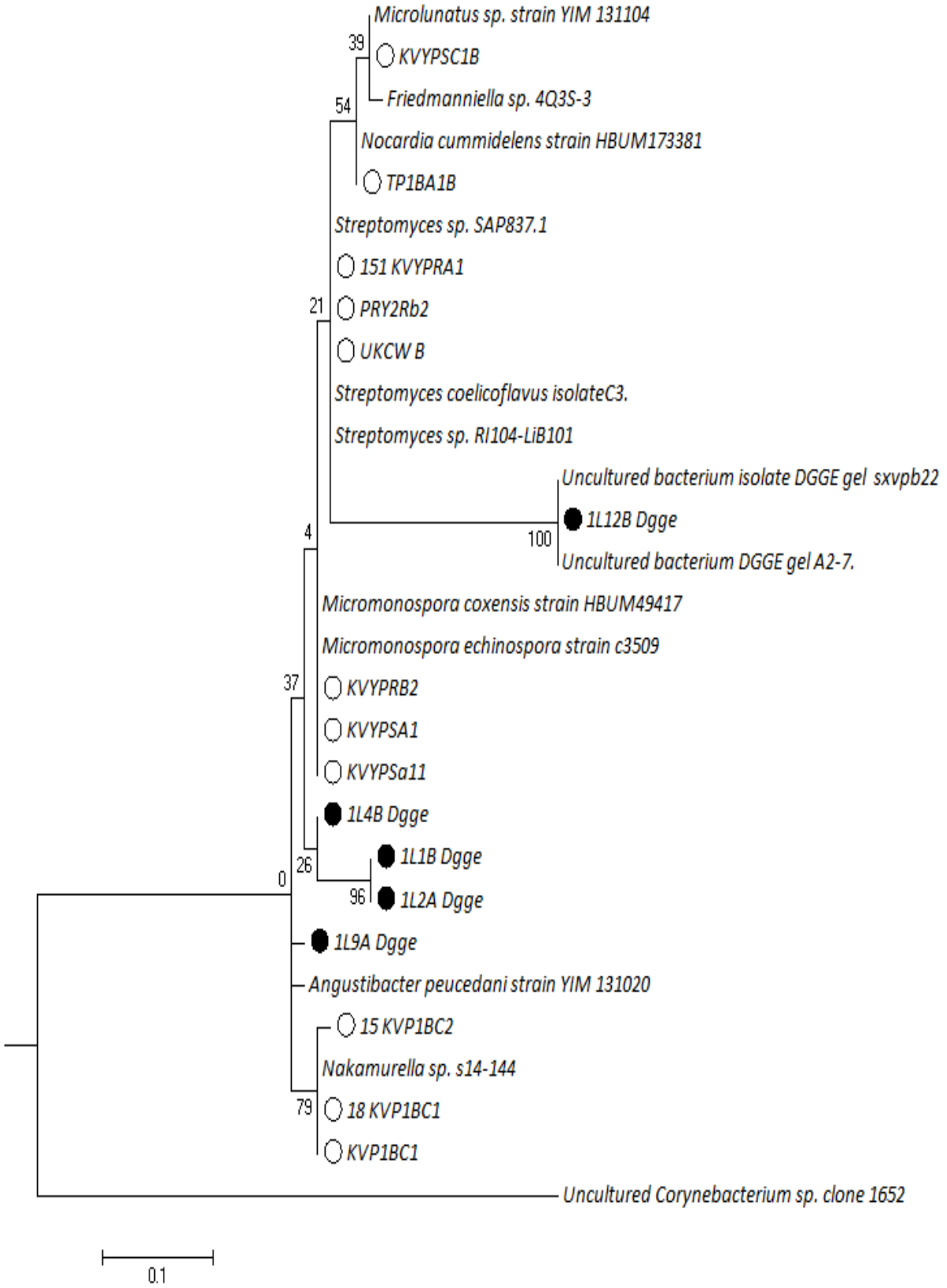


Figure 4.2: Phylogenetic tree based on alignment of partial 16S rRNA sequences of endophytic Actinobacteria associated with *Pseudowintera colorata* recovered as culturable isolates (O) and from DGGE gels (●).

Table 4.1: Percent similarity of the sequences of 16S rRNA gene from culturable endophytic Actinobacteria from *P. colorata* tissues and bands excised from DGGE gels.

Isolate	Type	Reference Strain (GenBank)	Similarity (%)	Accession no.
KVYPRB2	Cultured	*Micromonospora coxensis* strain HBUM49417	99	GQ163477.1
KVYPSA1	Cultured	*Micromonospora* sp. 29021/10ATCC1	99	JQ836673.1
KVYPSA11	Cultured	*Micromonospora* sp. I5	99	KC442361.1
KVYPRA1	Cultured	*Streptomyces* sp. RI104-Lib101	98	AB552918.1
UKCW/B	Cultured	*Streptomyces coelicoflavus* isolate c3	99	LN864567.1
PRY2RB2	Cultured	*Streptomyces* sp. SAP837.1	99	JX067713.1
KVP1BC1	Cultured	*Nakamurella* sp. s14-144	97	KX260107.1
TP1BA1B	Cultured	*Nocardia cummidelens* strain HBUM173381	99	FJ486303.1
KVYPSC1B	Cultured	*Microlunatus* sp. strain YIM 131104	98	KX502993.1
1L12B	DGGE	Uncultured bacterium isolate DGGE gel band sxvpb22	99	KC961606.1
1L9A	DGGE	*Angustibacter peucedani* strain YIM 131020	97	KX502961.1
1L4B	DGGE	Uncultured *Streptomyces* sp. isolate DGGE gel 10	95	LN649246.1
1L1B	DGGE	Uncultured bacterium clone ncd1250c09c1	100	JF114036.1
1L2A	DGGE	Uncultured bacterium clone ncd1250c09c1	100	JF114036.1

4.3.2 Activity against phytopathogenic fungi

Of the endophytic Actinobacteria strains tested, *Streptomyces* sp. PRY2RB2 showed highest activity against the four phytopathogenic fungi tested. *Micromonospora* sp. KVYPSA1 showed high activity against *I. liriodendri* WPA1C and *N. ditissima* ICMP 14417 (Fig. 4.3, Table 4.2).

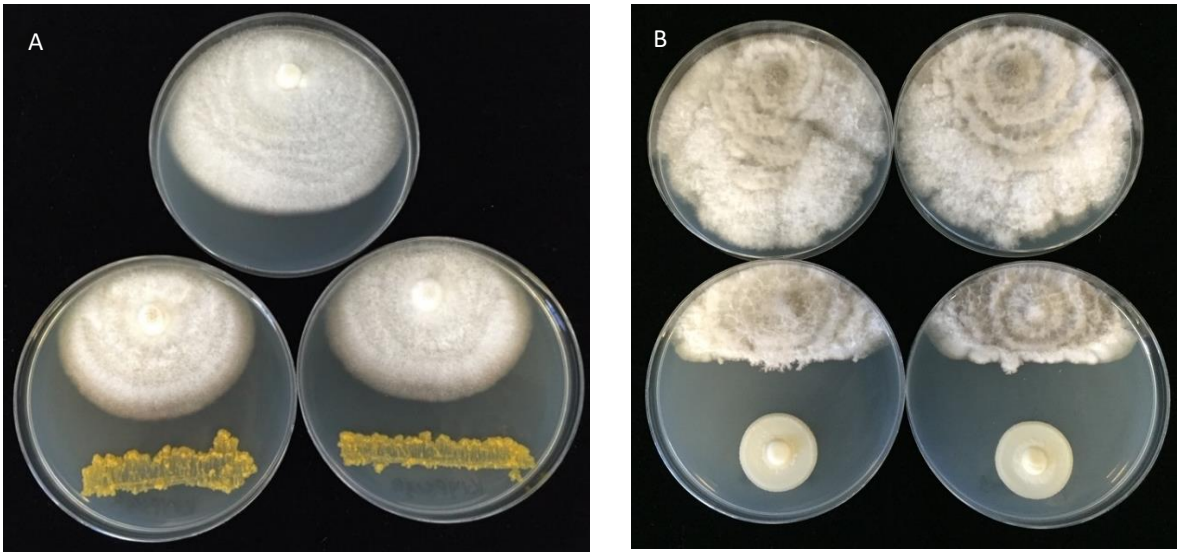


Figure 4.3: Plates showing the activity of endophytic Actinobacteria against phytopathogenic fungi. A) *Micromonospora* sp. KVYPSA1 against *Neonectria ditissima* ICMP 14417*,* B) *Streptomyces* sp. PRY2RB2 against *Neofusicoccum parvum* MM562*.* Top row: control, bottom row: plates with endophytic Actinobacteria and fungi.

Table 4.2 Activity of endophytic Actinobacteria against a range of fungal and bacterial phytopathogens and human pathogens, siderophore production on Chrom-azurol S agar (CAS) and phosphate solubilisation on tricalcium phosphate agar (TCP) and identification based on 16S rRNA sequencing.

Isolate	Tissue	*N. luteum*	*N. parvum*	*I. liriodendri*	*N. ditissima*	*P. atrosepticum*	*P. brasiliensis*	*S. aureus*	*E. coli*	*C. albicans*	CAS	TCP
Micromonospora sp. KVYPRB2	Root	-	-	-	-	-	-	-	-	-	NG	-
Micromonospora sp. KVYPSA1	Stem	-	-	+++	+++	-	-	-	-	-	NG	-
Micromonospora sp. KVYPSA11	Stem	-	-	-	-	-	-	-	-	-	NG	-
Microlunatus sp. KVYPSC1B	Stem	-	-	-	-	-	-	-	-	-	NG	-
Streptomyces sp. PRY2RB2	Root	+++	+++	+++	+++	++	-	+	-	-	+	
Streptomyces sp. UKCW/B	Root	-	-	-	-	-	-	-	-	-	+	++
Streptomyces sp. KVYPRA1	Root	-	-	-	-	-	-	+	-	-	NG	-
Nocardia sp. TP1BA1B	Stem	-	-	-	-	-	-	-	-	-	+	+
Nakamurella sp. KVP1BC1	Stem	-	-	-	-	-	-	-	-	-	NG	-

+++ High activity, ++ moderate activity, + low activity, - no activity, NG- No growth

4.3.3 Activity against phytopathogenic bacteria and opportunistic human pathogens

Only *Streptomyces* sp. PRY2RB2 showed activity against *P. atrosepticum*. None of the strains were active against *P. brasiliensis*, *E. coli* 916 and *C. albicans* 3395. *Streptomyces* sp. PRY2RB2 and *Streptomyces* sp. KVP1RA1 showed moderate activity only against *S. aureus* 297 (Fig. 4.4).

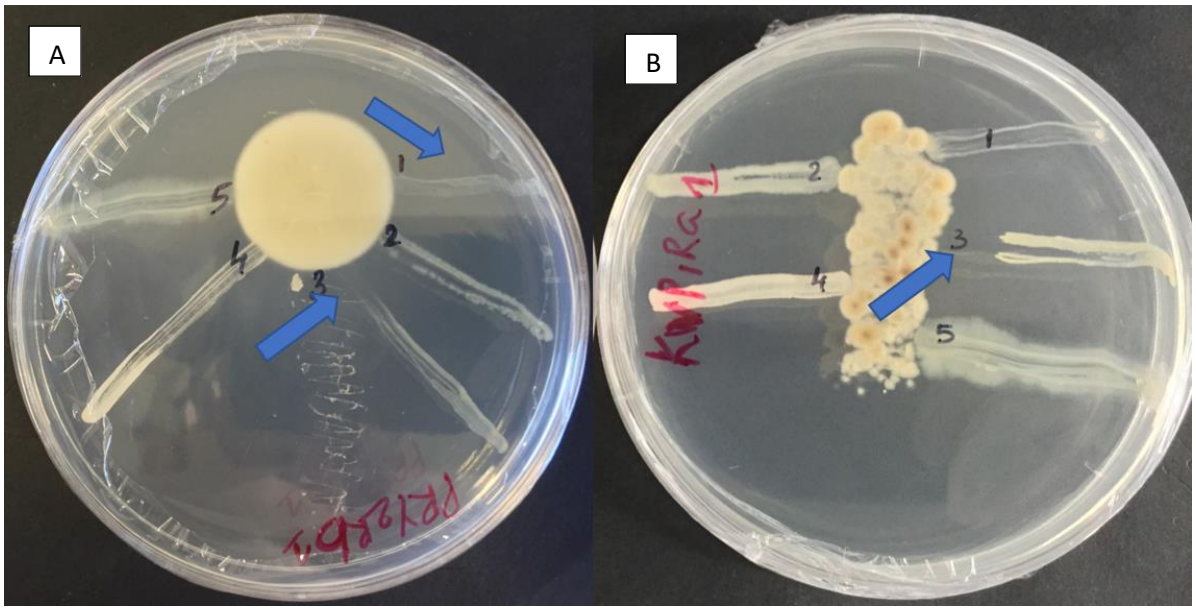


Figure 4.4: Activity of endophytic Actinobacteria against phytopathogenic bacteria and opportunistic human pathogens. Inhibition effect of (A) *Streptomyces* sp. PRY2RB2 and (B) *Streptomyces* sp. KVYPRA1. Numbers on plates indicate 1) *Pectobacterium atrosepticum,* 2) *P. brasiliensis,* 3) *Staphylococcus aureus,* 4) *Candida albicans* and 5) *Escherichia coli*. Arrows indicate clearance zones around colony.

4.3.4 Effect of secreted metabolites

The activity of the filtrates from *Streptomyces* sp. PRY2RB2 showed that plates with 10% filtrates inhibited *N. luteum* ICMP 16678 and *N. parvum* MM562 on day 2 but further incubation showed no difference between control and filtrate amended agar. There was no activity at both 1% or 10% concentration against *I. liriodendri* WPA1C, *N. ditissima* ICMP 14417 and the bacteria test pathogens in this study.

4.3.5 Production of siderophores

Of the strains tested (n=9) only *Streptomyces* sp. PRY2RB2, *Streptomyces* sp. UKCW/B and *Nocardia* sp.TP1BA1B produced faint halo zones (<1mm) on CAS agar (Table 4.2). Other test isolates failed to grow on CAS agar even after 3 weeks.

4.3.6 Phosphate solubilisation

On TCP agar, *Streptomyces* sp. UKCW/B produced a clearance zone greater than 4 mm (Fig. 4.5). *Nocardia* sp. TP1BA1B showed low activity by producing a faint clearance zone (< 1mm) (Table 4.2).

Figure 4.5: Growth of endophytic Actinobacteria, *Streptomyces* sp. UKCW/B on a TCP agar plate. Clearance zone around colony indicates solubilisation of phosphate.

4.4 Discussion

The endophytic Actinobacteria from medicinal plants are gaining international attention in pharmaceutical, agricultural and other industries (Trujillo *et al*., 2015; Golinska *et al.,* 2015). However, there are no published studies describing such endophytic Actinobacteria in New Zealand native plants. Endophytic Actinobacteria from medicinal plants have been reported as a major source of bioactive compounds with antimicrobial activity (Cao *et al*., 2004; Castillo *et al*., 2007). Kaewkla and Franco (2013), from their study on Australian native trees, indicated that native trees are potentially rich sources of high diversity and rare endophytic Actinobacteria genera. This is the first study reporting the diversity, isolation and bioactivity of endophytic Actinobacteria from a New Zealand native plant.

In this study, nine endophytic Actinobacteria belonging to five genera were isolated and cultured from the surface sterilized tissues of *P. colorata*. Despite results from Chapter 2

showing an average of 13 bands per lane for endophytic Actinobacteria in the DGGE gels, the number of culturable endophytic Actinobacteria isolated was comparatively low. Of the nine isolates, five were recovered from stem and four from roots. No culturable endophytic Actinobacteria were recovered from leaves despite Chapter 2 revealing leaves to be rich in Actinobacteria taxa. However, these results are consistent with results from chapter 3 where the least number of endophytes were recovered from leaves. The reason could be attributed to the presence of polygodial in the leaves, which the endophytes might have been exposed to during the recovery process and also the treatment with surface sterilizing agents which could easily enter the leaves through the stomata and thus affecting the overall recovery from leaves (Mercado-Blanco and Lugtenberg, 2014). However in this study, the effect of polygodial was not tested on the endophytes, but studies by Kubo *et al*. (2001, 2005) demonstrated activity of polygodial against bacteria like *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Salmonella typhi* and yeasts like *Candida albicans, C. utilis, C. krusei, Cryptococcus neoformans, Saccharomyces cerevisiae* and filamentous fungi including *Trichophyton mentogrophytes, T. ruburum* and *Penicillium marneffei*.

A preference for the recovery media and the natural antimicrobial nature of sequestered compounds in the tissues may also have affected the number of endophytic Actinobacteria recovered (Verma *et al.,* 2011; Mercado-Blanco and Lugtenberg, 2014). In order to increase the frequency of Actinobacteria isolated, researchers used several types of specific agar, for example Passari *et al*. (2015) used five different media for isolating endophytic Actinobacteria. In this study, only two media were used and these were Starch Casein agar and Bennet's agar amended with the antifungal agents Nystatin and cycloheximide (Costa *et al*., 2013; Passari *et al*., 2015). Qin *et al.* (2012) reported that media containing amino acids such as sodium propionate-arginine, cellulose-proline and xylan-arginine media were effective in isolation of endophytic Actinobacteria. The media used in this study did not contain these chemicals. For future studies, modifying and standardizing media would be crucial. Kaewkla and Franco, (2013) used ten different media to isolate Actinobacteria from four different Australian native trees and suggested slightly crushing the plant sample before plating it. This allowed the isolation of a total of 576 endophytic Actinobacteria belonging to genera *Streptomyces*, *Actinomadura*, *Gordonia*, *Micromonospora*, *Nocardia*, and

Pseudonocardia. To achieve better success in the recovery of culturable Actinobacteria from native plants it would be beneficial in future studies to use several different types of agar.

Sequencing the 16s rRNA gene of the culturable endophytic Actinobacteria revealed that the isolates belonged to the common genera *Streptomyces* sp. and *Micromonospora* sp. along with less common genera *Microlunatus* sp. and *Nakamurella* sp. Several studies have reported *Streptomyces* sp. and *Micromonospora* sp. as common endophytes in plants (Tian *et al*., 2007; Janso and Carter, 2010; El-Shatoury *et al*., 2013). The results of this study are consistent with the findings of El-Shatoury *et al*. (2013) investigating 10 different medicinal plants of Compositae family which revealed that 93% of the total isolated endophytic Actinobacteria (n=131) belonged to the genera *Streptomyces*, *Nocardiopsis* and *Micromonospora*. In addition to *Streptomyces* sp. and *Micromonospora* sp., other genera such as *Microlunatus* sp., *Nocardia* sp. and *Nakamurella* sp. were also recovered from *P. colorata* tissues. Sequencing some of the dominant bands from DGGE gels identified them as uncultured bacteria, uncultured *Streptomyces* sp. and *Angustibacter peucedani*. The uncultured bacteria may represent novel Actinobacteria present in *P. colorata* that could not be resolved due to the small size of the sequence obtained. Additional work by cloning these bands may produce a better sequence and identification in future. Of the ten DGGE bands that were successfully sequenced, 50% of them were identified as chloroplast, which could also explain the differences between the number of bands shows in chapter 2 and the number of Actinobacteria actually cultured in this study.

In this study, the diversity of culturable endophytic Actinobacteria in the tissues of *P. colorata* was lower compared to the other microbial groups. Although these are not likely to be the complete community of Actinobacteria in *P. colorata* these findings are in accordance with the NGS analysis from Chapter 2 which showed that only 1.2% of phyla belonged to Actinobacteria. Together these results indicate a low diversity of Actinobacteria *in planta,* of which < 5% were culturable. Results for the number of culturable Actinobacterial species were consistent with Passari *et al*. (2015) where a total of 42 endophytic Actinobacteria belonging to 5 genera were isolated from the tissues (roots n=22, stems n=9, leaves n=6, flower n=3 and petiole n=2) of seven different medicinal plants (shrubs and trees) in India, with *Mirabilis jalapa* yielding the highest number of endophytic Actinobacteria (n=12).

International research groups have demonstrated that endophytic Actinobacteria can inhibit phytopathogens through production of antifungal compounds (Coombs *et al*., 2004), and siderophores (Cao *et al*., 2005) in addition to promoting plant growth (El-Tarabily *et al*., 2009). In this study, a *Streptomyces* sp. was active against all the phytopathogenic fungi tested. The other species which showed activity against phytopathogenic bacteria/fungi and opportunistic human pathogens were members of genera *Streptomyces* and *Micromonospora*. *Streptomyces* spp. are known to produce several bioactive metabolites and antibiotics (Berdy, 2012). For example, commercial products such as Mycostop® and Actinovate® are products containing *Streptomyces* sp. and are used in controlling damping-off caused by *Rhizoctonia solani* in tomato plants (Goudjal *et al*., 2014). Several research groups have shown the potential of *Streptomyces* sp. and *Micromonospora* sp. as biocontrol agents and plant growth promoters. El-Tarabily *et al*. (2009) showed that endophytic Actinobacteria isolated from cucumber roots, *Streptomyces spiralis* and *Micromonospora chalcea,* were highly active against *Pythium aphanidermatum*. Verma *et al*. (2011) reported that the endophytic *Streptomyces* sp. isolated from the Indian medicinal plant *Azadiractha Indica* showed strong antifungal activity against *Alternaria alternata in vitro.*

For siderophore production, three isolates showed faint orange halos on CAS agar. The faint halos on CAS agar suggest that production of siderophores is not a major mechanism involved in the activity against phytopathogenic fungi. These results were consistent with El-Tarabily *et al*. (2009), where the endophytic Actinobacteria tested did not produce siderophores on CAS agar but were active against *P. aphanidermatum*.

In addition to producing siderophores, endophytic Actinobacteria are known to promote plant growth by solubilizing phosphate. Of the endophytic Actinobacteria tested in this study, *Streptomyces* sp. UKCW/B produced a clear zone greater than 3 mm on TCP agar. *Nocardia* sp. TP1BA1B produced a faint clearance zone. These results were similar to Verma *et al*. (2011) where the endophytic *Streptomyces* sp. isolated from *A. indica* solubilized phosphate on TCP agar. The strains (n=2) which were able to solubilize phosphate on TCP agar were further tested for their effect on *P. colorata* growth in Chapter 5.

In conclusion, the results of this chapter showed that *P. colorata* is host to endophytic Actinobacteria with potential as biocontrol agents and plant growth promoters. These findings support the results from Chapter 2 and provide an insight into the diversity and

bioactive potential of endophytic Actinobacteria within the tissues of *P. colorata*. The functional relationship between these Actinobacteria and their host is yet to be tested, however this research has demonstrated that several strains possess bioactivity *in vitro*.

Chapter 5

Effects of endophytes on the growth of *Pseudowintera colorata*

5.1 Introduction

Endophytes can directly or indirectly influence plant growth and productivity. Taurian *et al*. (2010) demonstrated that inoculation of a phosphate solubilizing endophytic bacterium, *Pantoea agglomerans,* in peanut improved growth and induced greater nodulation in the host. Several studies have shown that endophytes have the ability to colonize internal plant tissues and promote plant growth in addition to enhancing host stress tolerance (Saravanakumar and Samiyappan, 2007; Li *et al.,* 2016a). Li *et al.* (2016a) demonstrated that inoculation of endophytic bacteria belonging to genera *Pantoea*, *Bacillus*, *Enterobacter* and *Sphingomonas* in the Hybrid *Pennisetum* significantly increased biomass yield in addition to alleviating the harmful effects of salt stress on the host plant. A study by Tsavkelova *et al*. (2007) demonstrated that addition of the auxin producing endophytic bacteria *Pseudomonas* sp. and *Stenotrophomonas* sp. stimulated the root development and growth of kidney bean plants.

Medicinal plants are renowned for their essential oils and bioactive metabolites, of which isoprenoids are the largest and structurally most diverse group (Withers and Keasling, 2007). Isoprenoids are involved in functions like photosynthesis (chlorophyll) and growth regulation (gibberellic acid, cytokinins) (Chappell, 1995; Wanke *et al*., 2001). Sesquiterpenes are the most diverse class of isoprenoids and are constituents of several plant essential oils. Leaves of *P. colorata* contain two sesquiterpene dialdehydes, polygodial and 9-deoxymuzigadial (Mc Callion *et al.*, 1982; Gerard *et al.*, 1993). *Pseudowintera colorata* leaves are utilised for commercially extracting polygodial, which is used in the product Kolorex® (Forest Herbs Research) for treating intractable yeast infections. However, for large scale production of many sesquiterpenes, extraction from plants is not sustainable or feasible (Asadollahi *et al*., 2008). In addition, problem of using plants such as *P. colorata* as the source include dealing with slow growth. Thus endophytic inoculants may offer a solution and may promote the growth of *P. colorata* which could have a potential impact on the overall ecology and chemistry of the host.

In the last decade, numerous studies have been conducted using microorganisms as cell factories for production of biologically important compounds, including, isoprenoids (Maury *et al*., 2005). In addition, other research groups have shown that some endophytes are capable of producing the same biologically active compounds as the hosts in which they reside. For example, the endophytic fungus *Taxomyces andreanae* isolated from *Taxus brevifolia,* and *Periconia* sp. isolated from *Piper longum,* are capable of producing paclitaxel (Taxol®) and piperine, respectively, the same compounds produced by their plant hosts (Stierle *et al*., 1993; Verma *et al*., 2011). To date there are no published studies of production of polygodial from any microbial sources.

Thus the aims of this chapter were two fold. Firstly to determine whether the endophytes recovered from *P. colorata* influenced plant growth when reintroduced in high numbers and secondly, to determine whether polygodial could be produced by microbial endophytes. For the second aim it was recognized that polygodial may not be formed by endophytes or that it might require more than one endophyte and/or interaction with the plant itself. It was hypothesised that such biotransformation was more likely by fungal endophytes (Nigel Perry pers. comm.) as they are recognized as multi-enzyme systems capable of performing a wide variety of chemical transformations of different starting materials such as Farnesol.

Farnesol is a natural 15-carbon acyclic sesquiterpene alcohol which is produced as a by-product of the ergosterol biosynthesis pathway (Nickerson *et al*., 2006). Farnesol exists widely in fruits such as peaches, vegetables like tomatoes and corn, herbs such as chamomile and in the essential oils of ambrette seeds and citronella (Ku and Lin, 2015). Research by Nankai *et al*. (1998) and Gliszczýnska and Wawrzénczyk, (2008) successfully showed that fungi could biotransform farnesol into cyclic compounds such as homogeraniol and dihydroxygeranylacetone. In addition to fungi, the endophytic bacteria which showed activity against *C. albicans* in chapter 3 were also tested.

5.2 Materials and Methods

5.2.1 Influence of endophytic fungi, bacteria and Actinobacteria on the growth of *P. colorata* seedlings in the glasshouse

In this study, the effect of endophytic fungi, Actinobacteria and bacteria on the growth of *P. colorata* was tested. Six week old seedlings were purchased from Southern Woods Plant

Nursery (Christchurch, New Zealand). The seedlings were not from the same seed lot and, at the time of purchase, the seedlings did not yet have a fully developed root system. The seedlings were acclimatized in the shade house for 3-4 weeks in February 2017 before setting up the experiment. Immediately prior to inoculation with the endophyte treatments the length of the shoots and the stem girth were measured using a digital calliper.

Experiment 1: Inoculation of endophytic Actinobacteria and Bacteria

The Actinobacteria cultures were prepared by first reviving the test isolates from -20°C cold storage onto starch casein agar and these plates were incubated at 25°C for 7-10 d in the dark. After the colonies emerged, a sterile cork borer was used to take 6 mm plugs (n=2) of each strain that were inoculated into separate flasks each containing 150 mL of sterile Waksman broth (WB). The flasks were incubated in a shaking incubator (Labnet 211DS) set at 25°C and 150 rpm in the dark for 5-7 d. The cultures were harvested by centrifuging at 20,000 X *g* at 4°C for 15 min to pellet the cells. The supernatant was discarded and the pellet was resuspended in autoclaved distilled water. The spore concentration of the suspension was determined using a haemocytometer and adjusted to 1 x 10^6 cells/ mL using sterile distilled water (SDW).

Endophytic bacteria were revived from -80°C onto nutrient agar (NA, Difco) plates and the plates were incubated at 25°C in total darkness for 2-3 d. After 2-3 d, a single colony of the isolates was then picked using a sterile inoculation loop and inoculated in a 50 mL tube (Axygen, USA) containing 30 mL nutrient broth (NB, Difco). The tubes were incubated in a shaking incubator set at 25°C and 200 rpm in the dark for 2 d. Bacterial cells were harvested by centrifugation at 20,000 X *g* at 4°C for 10 min to pellet the cells. The supernatant was discarded and the pellet was suspended in SDW. The final concentration of the bacteria was adjusted to 1 x 10^6 to 10^7 cells/ mL by plate dilution and measuring OD (optical density).

Experiment 2: **Inoculation of endophytic fungi**

The endophytic fungi were revived from -80°C onto PDA plates and incubated at 25°C for 5-7 d in 12 h light/12 h dark regimes. When the fungal mycelia had covered more than half of the agar surface, 1-2 mL SDW was added to the plates. Using a sterile glass spreader, the spores were dislodged and the spore suspension was added to a 50 mL tube. The spore concentration

of the suspension was determined using a haemocytometer and adjusted to 1 x 10^5 cells/mL in a total volume of 1 L of SDW in aluminium trays.

Prior to inoculation with the fungal, bacterial or Actinobacterial cultures, the *P. colorata* seedlings were not watered for 24-48 h. Each seedling being treated with either a bacterial or Actinobacterial inoculum was transferred to a 1 L pot containing the potting mix medium on the day of inoculation. The potting mix was composed of 20% pumice, 80% composted bark, 2kg/m^3 Osmocote® standard 3-4 months gradual release fertilizer (NPK 16-3.5-10 plus trace elements), 1 kg/ m^3 agricultural lime, 500 g/m^3 Hydraflo® 2 (granular wetting agent, Scott Australia Pty Ltd, Auckland, New Zealand). Using a sterile pipette, the root region of *P. colorata* seedlings was drenched with 50 mL of the appropriate cell suspension. For control plants, sterile distilled water without any cell suspension was added.

For inoculation with the fungal spore suspensions, the seedlings of *P. colorata* with their soil-plugs intact were carefully soaked in the spore suspensions overnight in aluminium trays, with the trays being covered to avoid evaporation or cross contamination. The following day, the seedlings with the soil-plugs were then repotted into 1L pots containing potting mix medium. Each treatment was replicated 10 times and there were 10 uninoculated control *P. colorata* seedlings. The control seedlings were soaked in SDW. The pots were arranged in a randomised complete block design using randomisation generated by research randomizer (https://www.randomizer.org/). The plants were watered once daily from the following day and observed regularly to see if there were any dead or diseased plants following the treatments. The shoot height of the seedlings was measured after 3 months (March 2017 to May 2017) and the difference in heights pre-treatment (X) and post-treatment (Y) was calculated (Y-X).

After 3 months growth, using a sterile pipette, the potting mix around the root region of each treatment of *P. colorata* seedlings was reinoculated by drenching with 50 mL of freshly prepared spore suspensions of each of the respective treatments. The spore concentration was adjusted to 1 x 10^5 cells/ mL for fungi and 1 x 10^6 to 10^7 cells/ mL for bacteria and Actinobacteria using SDW. The seedlings were destructively harvested 4 weeks after the second inoculation (June 2017).

At harvest the shoot height was measured from the stem base (at the soil level) to the top leaf using a digital calliper. The number of internodes was measured for each plant stopping at the top 2 leaves. The shoot and root portions were weighed after drying to a constant weight in an oven at 60°C for 2 d. The data were analysed using a general analysis of variance (ANOVA). Fisher's protected least significant difference (LSD) was used to test the mean difference between shoot lengths, shoot weights and root weights of treated plants with untreated controls. The analyses were performed in Minitab 17 (Lead Technologies, Australia).

5.2.2 Studying the influence of the endophytes on the microbial communities in the roots using DGGE

To identify any effects of the inoculants on the microbial communities within the roots of *P. colorata* seedlings, roots from the three seedlings for each treatment that showed the maximum growth were selected. The roots were surface sterilized as described in Section 2.1.3 and DNA was extracted from the roots as described in Section 2.1.4. PCRs were performed with group specific primers for Actinobacteria, Alpha, Beta and Gammaproteobacteria and total fungi as described in Sections 2.1.5.1 to 2.1.5.4. DGGE and analysis of the gels and statistics was done as described in Section 2.1.6. To confirm if the endophytes were able to colonize the roots of *P. colorata* seedlings, isolates used for inoculation studies were used as reference markers in DGGE gels. The DNA from pure cultures was amplified using the primers as described in Sections 2.1.5.1 and 2.1.5.4.

5.2.3 Potential of endophytic fungi to biotransform Farnesol

The biotransformation potential of endophytic fungi (n=7) and endophytic bacteria (n=1) (Chapter 3) which showed activity against *C. albicans* in plate assays were tested in this study. The protocol from Gliszczýnska and Wawrzénczyk (2008) was modified and adopted. The endophytic bacterium and fungi were revived from -80°C onto NA and PDA plates, respectively, and incubated as per the conditions described in Section 5.2.1. After 24-48 h, using a sterile inoculation loop, a single colony of the bacterium was added to a conical flask containing 150 mL sterile WB. After the fungal colonies covered about half of the plate, using a sterile cork borer, a 6 mm plug from the margins of each colony was transferred into separate sterile conical flasks each containing 150 mL WB. The flasks inoculated with the

cultures were incubated in a shaking incubator (Labnet 211DS) set at 25°C and 150 rpm in the dark. After 2-5 d of growth, 10 mg of farnesol (Sigma-Aldrich) in 1 mL of acetone (Sigma-Aldrich) was added to each flask. Positive control flasks containing WB with test bacteria and fungi only and negative control flasks containing WB with farnesol were set up.

After addition of farnesol, the flasks were incubated for a further 5-7 d. The flasks were prepared in triplicates. To test if the presence or absence of plant material influenced the formation of anti-yeast compounds, 15-20 surface sterilized leaves of *P. colorata* were macerated using a blender. About 100 mL water was added to the macerate. The macerate was autoclaved and cooled to 30°C in a water bath. One mL of the macerate was added to conical flasks each containing WB and a fungal or bacterial endophyte. Positive controls consisted of WB and test isolate without any Farnesol. Negative control flasks containing WB only, WB with farnesol only and WB with both farnesol and leaf macerate were also set up.

For time-course analysis, 10 mL of the incubation mixture was taken after 3 d, 5 d, and 7 d and centrifuged at 20,000 X *g* at 4°C for 15 min to pellet the mycelia. The supernatant was then filter sterilized using a 0.22-micron pore size filter. *Candida albicans* cultures were grown overnight in 2 mL tubes containing NB incubated at 25°C. After 24 h, 100 μL of the overnight culture was spread onto WA plates using a sterile spreader. Using a sterile 6 mm cork borer, wells were made in the middle of the plates. To these wells, 200 μL of the filter sterilized supernatant from respective treatments (including positive and negative controls) was added. The plates were prepared in triplicates. For the negative control plates, the wells were inoculated with sterile NB. The plates were sealed and incubated at 25°C and were observed daily for 5-7 d. Presence of a clear zone around the well was noted as positive and the results were recorded in comparison to a negative and positive control plates.

5.3 Results

5.3.1 Effect of endophytic Actinobacteria, bacteria and fungi on the growth of *P. colorata* seedlings

5.3.1.1 Actinobacteria and bacteria

Inoculation of endophytic bacteria and Actinobacteria increased the growth of *P. colorata* seedlings for all the treatments compared to the control ($P<0.05$, Appendix D.1) (Table 5.1, Fig. 5.1). Shoot height of seedlings treated with *Pantoea* sp. AP1SA1 were 1.8 × longer (5.79

cm) than the control (3.12 cm) but were not different from the other three treatments (range = 4.93 to 5.71 cm). Shoot dry weight of seedlings treated with *Bacillus* sp. TP1LA1B and *Nocardia* sp. TP1BA1B were 1.8 and 1.6 × times heavier (1.38 g and 1.22 g, respectively) than that of the control (0.76 g). Root dry weight of seedlings treated with *Nocardia* sp. TP1BA1B was 1.6 x times heavier (0.73 g) than the control (0.46 g) but were not different from seedlings treated with *Bacillus* sp. TP1LA1B (0.69 g) and *Streptomyces* sp. UKCW/B (0.61 g) (Table 5.1). Number of internodes produced by the treated plants were significantly higher compared with the control, with *Bacillus* sp. TP1LA1B producing 1.8 × (6.8) more internodes than the control (3.7), but not significantly different from *Pantoea* sp. AP1SA1 (6.7) (Table 5.1).

Table 5.1 Response of *Pseudowintera colorata* seedlings following treatment with endophytic bacteria and Actinobacteria after 4 months growth. Mean of 10 replicate plants per treatment

Treatment	Shoot height (cm)	Shoot dry weight (g)	Root dry weight (g)	Number of internodes
Pantoea sp.AP1SA1	5.79 a[1]	0.79 cd	0.47 b	6.7 ab
Bacillus sp. TP1LA1B	5.71 a	1.38 a	0.69 a	6.8 a
Nocardia sp. TP1BA1B	5.17 a	1.22 ab	0.73 a	6.2 b
Streptomyces sp. UKCW/B	4.93 a	1.07 cd	0.61 ab	4.7 c
Negative Control	3.12 b	0.76 d	0.46 b	3.7 d
P Value	0.016	<0.001	0.007	<0.001
LSD (5%)	1.664	0.290	0.175	0.592

[1]Means within a column followed by the same letter are not significantly different based on least significant difference (LSD) at P=0.05

Figure 5.1: Pots showing the difference in height of *Pseudowintera colorata* seedlings treated with *Streptomyces* sp. UKCW/B and the negative control.

5.3.1.2 Fungi

Inoculation of the endophytic fungi significantly increased the growth of *P. colorata* seedlings for all the treatments compared to the control ($P<0.05$, Appendix D.2) (Table 5.2, Fig. 5.2) except for *Metarhizium* sp. PR1SB1. Shoots of seedlings treated with *Trichoderma* sp. PRY2BA21 were 2.2 × longer (8.36 cm) than the control (3.72 cm) but were not significantly different from seedlings treated with *Trichoderma* sp. PRY3BC1, *Chaetomium* sp. PR1BC2, *Xylaria* sp. P4BB2 and *Fusarium* sp. P4LC2 (Table 5.2). Shoot and root weights of the treated seedlings were not significantly different from that of the control ($P=0.88$ and $P=0.31$, respectively) (Table 5.2). Treatment with *Fusarium* sp. P4LC2 produced significantly more internodes (mean=7) compared with all other treatments (means=6.0-4.1) ($P<0.005$). Of these, treatment with *Trichoderma* sp. PRY2BA21, *Chaetomium* sp. PR1BC2, *Xylaria* sp. P4BB2 and P4LA3 produced significantly more internodes compared with the untreated control.

Table 5.2 Response of *Pseudowintera colorata* seedlings to treatment with endophytic fungi after 4 months growth. Mean of 10 replicate plants per treatment

Treatment	Shoot height (cm)	Shoot dry weight (g)	Root dry weight (g)	Number of internodes
Trichoderma sp. PRY2BA21	8.36 a[1]	1.14	0.68	6.0 b
Trichoderma sp. PRY3BC1	7.46 ab	0.95	0.72	4.8 cd
Chaetomium sp. PR1BC2	7.35 ab	0.98	0.54	6.0 b
Xylaria sp. P4BB2	6.84 ab	0.99	0.62	5.3 bc
Fusarium sp. P4LC2	6.79 ab	1.1	0.59	7.0 a
Xylaria sp. P4LA3	5.97 bc	0.93	0.69	5.8 b
Metarhizium sp. PR1SB1	4.99 cd	1.1	0.47	4.3 d
Untreated Control	3.72 d	1.02	0.59	4.1 d
P Value	<0.001	0.88	0.31	<0.001
LSD	1.771	NSD	NSD	0.816

[1]Means within a column followed by the same letter are not significantly different based on least significant difference (LSD) at P=0.05, NSD- not significantly different.

Figure 5.2: Pots showing the difference in height of *Pseudowintera colorata* seedlings treated with *Trichoderma* sp. PRY2BA21 and untreated control.

5.3.2 Influence of the endophytic inoculants on the microbial communities in the roots of *P. colorata*

The influence of inoculating endophytic bacteria, Actinobacteria and fungi on the microbial communities in the root tissues of the seedlings of *P. colorata* was analysed using DGGE. In this study, the control seedlings from experiment 1 and experiment 2 were treated as one group for further analysis. In addition, analysis in PRIMER 7 for individual treatments did not form any grouping pattern, hence the nMDS analysis was done looking at the effects of microbial group as a whole (bacteria, fungi and Actinobacteria).

5.3.2.1 Actinobacteria

Inoculation of the seedlings with *Trichoderma* sp. PRY3BC1 decreased the diversity of Actinobacteria communities in the roots of *P. colorata* seedlings (PERMANOVA *P*=0.05) (Table 5.3). Other inoculants did not have any influence on the Actinobacteria communities (Table 5.3). There was no clustering observed within different treatments (Fig. 5.3). Treatments of seedlings with *Bacillus* sp. TP1LA1B produced more DGGE bands (n=25), whereas, treatment with *Metarhizium* sp. PR1SB1 produced less DGGE bands (n=12) compared to the control (n=18) and, thus, influenced the richness of Actinobacteria in the roots of *P. colorata* seedlings (*P*=0.004 and *P*=0.016, respectively) (Table 5.4) (Appendix D.3).

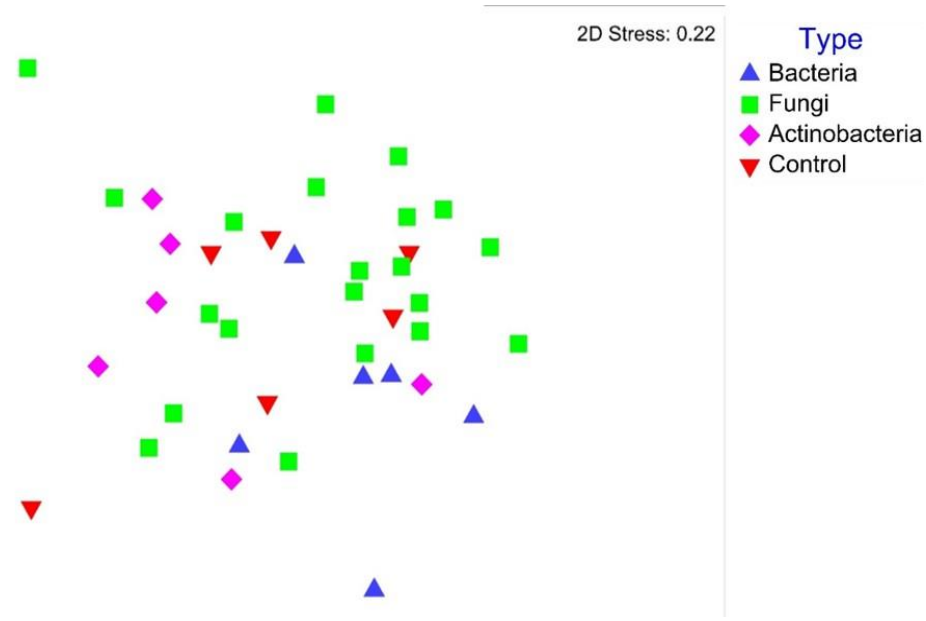


Figure 5.3: Nonmetric multidimensional scaling (nMDS) plot showing Actinobacteria communities in the roots of *P. colorata* seedlings with different endophyte inoculation treatments.

Table 5.3 Effect of endophytic inoculants on the similarity of microbial communities in the root tissues of seedlings of *Pseudowintera colorata*

Treatment	†Microbial communities similarity				
	Actinobacteria	α proteobacteria	β proteobacteria	γ proteobacteria	Total Fungi
Trichoderma sp. PRY2BA21	0.65	0.85	0.12	0.45	0.17
Trichoderma sp. PRY3BC1	0.05*	0.17	0.17	0.13	0.18
Chaetomium sp. PR1BC2	0.79	0.70	0.45	0.43	0.97
Xylaria sp. P4BB2	0.28	0.71	0.98	0.25	0.33
Fusarium sp. P4LC2	0.68	0.78	0.98	0.51	0.16
Xylaria sp. P4LA3	0.62	0.73	0.91	0.59	0.11
Metarhizium sp. PR1SB1	0.10	0.72	0.84	0.57	0.24
Pantoea sp.AP1SA1	0.58	0.62	0.52	0.60	0.54
Bacillus sp. TP1LA1B	0.28	0.82	0.52	0.15	0.72
Nocardia sp. TP1BA1B	0.23	0.54	0.48	0.93	0.47
Streptomyces sp. UKCW/B	0.49	0.37	0.11	0.33	0.59

Table 5.4 Effect of endophytic inoculants on the microbial richness in the root tissues of seedlings of *Pseudowintera colorata*

Treatment	†Microbial community richness				
	Actinobacteria	α proteobacteria	β proteobacteria	γ proteobacteria	Total Fungi
Trichoderma sp. PRY2BA21	0.513	0.34	0.13	0.97	0.44
Trichoderma sp. PRY3BC1	0.991	0.17	0.07	0.76	0.03*
Chaetomium sp. PR1BC2	0.185	0.92	0.47	0.42	0.27
Xylaria sp. P4BB2	0.817	0.65	0.41	0.25	0.44
Fusarium sp. P4LC2	0.798	0.10	0.54	0.89	0.96
Xylaria sp. P4LA3	0.681	0.38	0.44	0.58	0.22
Metarhizium sp. PR1SB1	0.016*	0.42	0.44	0.97	0.59
Pantoea sp.AP1SA1	0.196	0.94	0.93	0.16	0.06
Bacillus sp. TP1LA1B	0.004**	0.11	0.89	0.29	0.46
Nocardia sp. TP1BA1B	0.113	0.60	0.57	0.41	0.39
Streptomyces sp. UKCW/B	0.868	0.70	0.76	0.29	0.86

†Asterisk denotes levels of statistical significance of microbial communities richness based on GLM in comparison to untreated control seedlings. *significantly different ($P \leq 0.05$), **high significant difference ($P \leq 0.005$)

5.3.2.2 Alphaproteobacteria

Inoculation of *P. colorata* seedlings with endophytic bacteria, fungi and Actinobacteria did not influence the Alphaproteobacteria community diversity or richness in the roots (Table 5.3 and Table 5.4). Treatment with Actinobacteria and fungi produced similar bands and clustered together while the treatments with bacteria and controls were more diverse (Fig. 5.4).

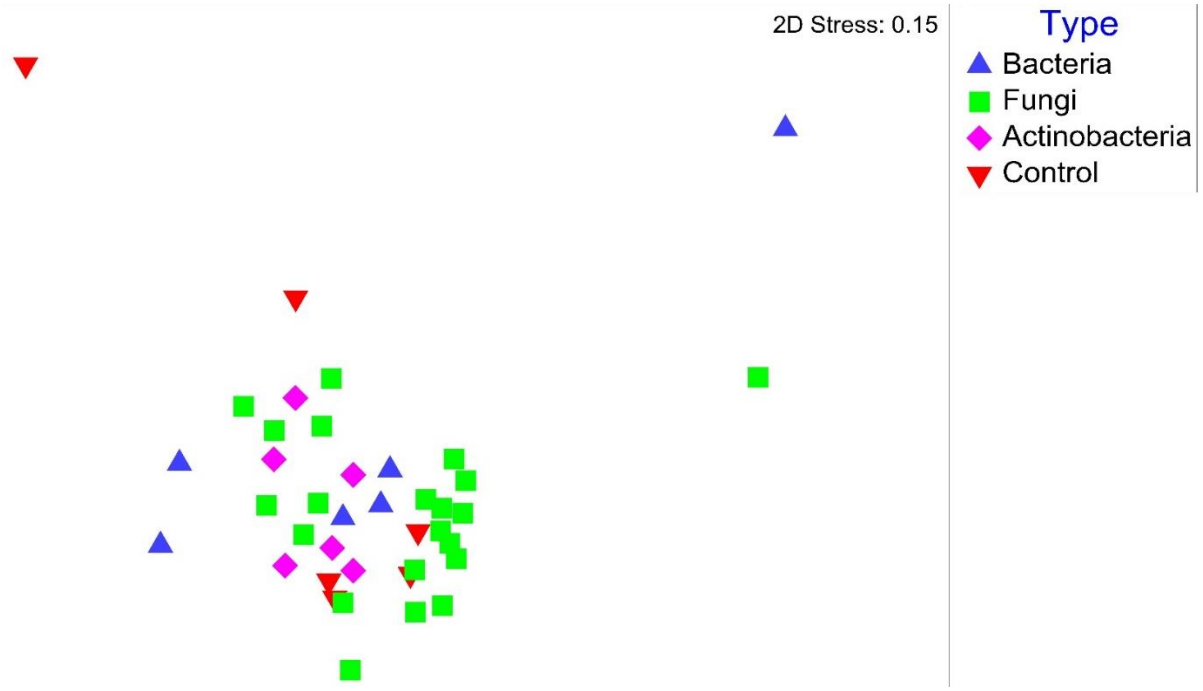


Figure 5.4: Nonmetric multidimensional scaling (nMDS) plot showing Alphaproteobacteria communities in the roots of *P. colorata* seedlings with different endophyte inoculation treatments.

5.3.2.3 Betaproteobacteria

Inoculation of *P. colorata* seedlings with endophytic bacteria, fungi and Actinobacteria did not influence the Betaproteobacteria community diversity or richness in the roots. (Table 5.3 and Table 5.4). There was no clustering observed among any of the treatments (Fig. 5.5).

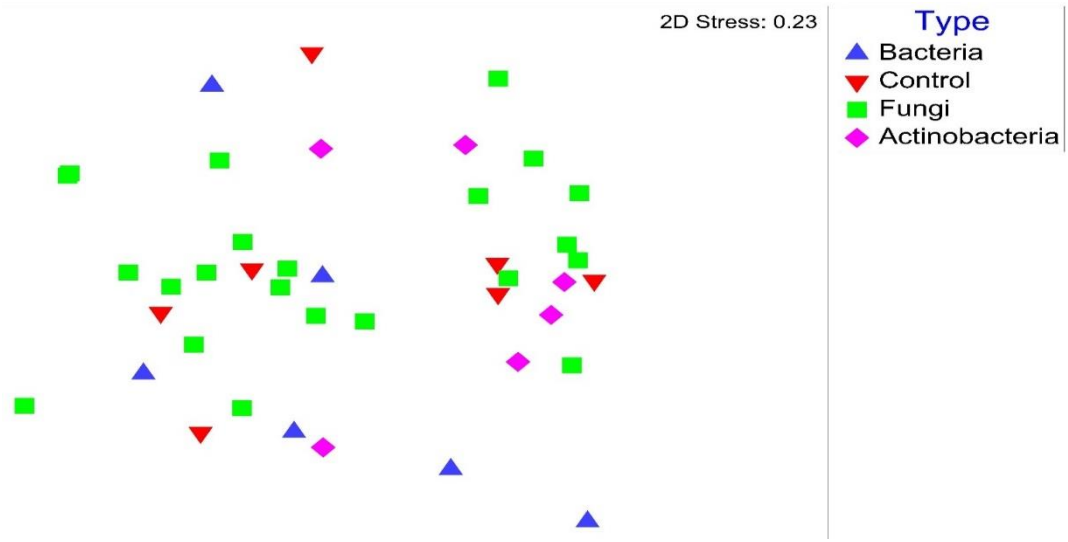


Figure 5.5: Nonmetric multidimensional scaling (nMDS) plot showing Betaproteobacteria communities in the roots of *P. colorata* seedlings with different endophyte inoculation treatments.

5.3.2.4 Gammaproteobacteria

Inoculation of *P. colorata* seedlings with endophytic bacteria, fungi and Actinobacteria did not influence the Gammaproteobacteria community diversity or richness in the root tissues (Table 5.3 and Table 5.4). Treatments with Actinobacteria formed discrete clusters while the treatments with fungi, bacteria and control were diverse (Fig. 5.6).

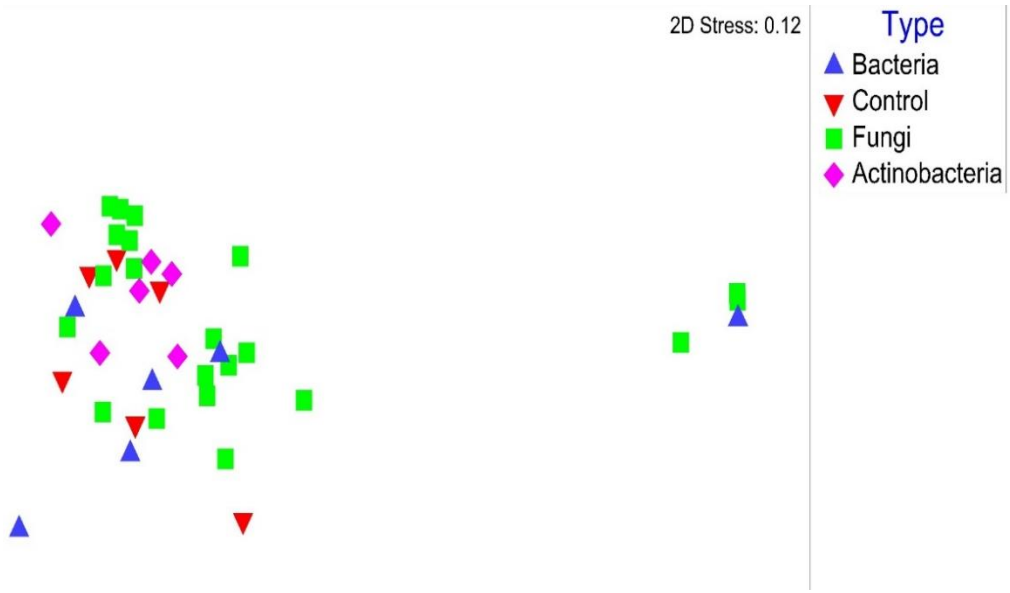


Figure 5.6: Nonmetric multidimensional scaling (nMDS) plot showing Gammaproteobacteria communities in the roots of *P. colorata* seedlings with different endophyte inoculation treatments.

5.3.2.5 Total fungi

Treatment of *P. colorata* seedlings with endophytic bacteria, Actinobacteria and fungi did not influence the diversity of the total fungi communities in the roots of *P. colorata* seedlings (Table 5.3 and Table 5.4) (Fig. 5.7). Treatment with *Trichoderma* sp. PRY3BC1 increased the richness of the total fungi in the root tissues of *P. colorata* seedlings with an average of 23 bands (n=3) (P<0.05) (Table 5.4) compared to the control (16) and other treatments (range 13-18) (Appendix D.4).

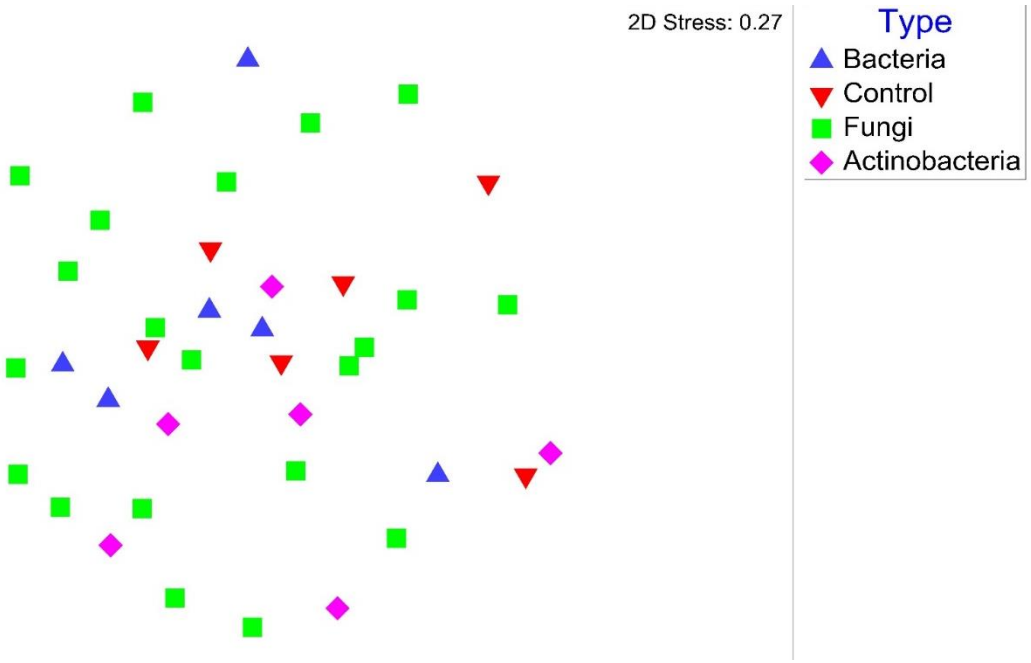


Figure 5.7: Nonmetric multidimensional scaling (nMDS) plot showing total fungal communities in the roots of *P. colorata* seedlings with different endophyte inoculation treatments.

Colonization of *P. colorata* roots by endophytes as shown by DGGE

The endophytic colonization of the roots by the isolates used for inoculation studies was confirmed using DNA from the inoculated isolates as reference markers on DGGE. Bands corresponding to Actinobacteria isolates *Nocardia* sp. TP1BA1B and *Streptomyces* sp. UKCW/B were identified in the treatment lanes and thus confirmed colonization (Fig. 5.8) However, there was no attempt to re-isolate the endophytes onto agar from the tissues of the *P. colorata* seedlings. For fungi, only bands corresponding to *Metarhizium* PR1SB1, *Xylaria* sp. P4LA3 and *Fusarium* sp. P4LC2 were identified in the respective treatments (Fig. 5.8). *Xylaria* sp. P4LA3 showed complex profile with 2 bands (Fig. 5.8 Lanes 17-20). Bands for other fungal isolates were not observed in the treatments.

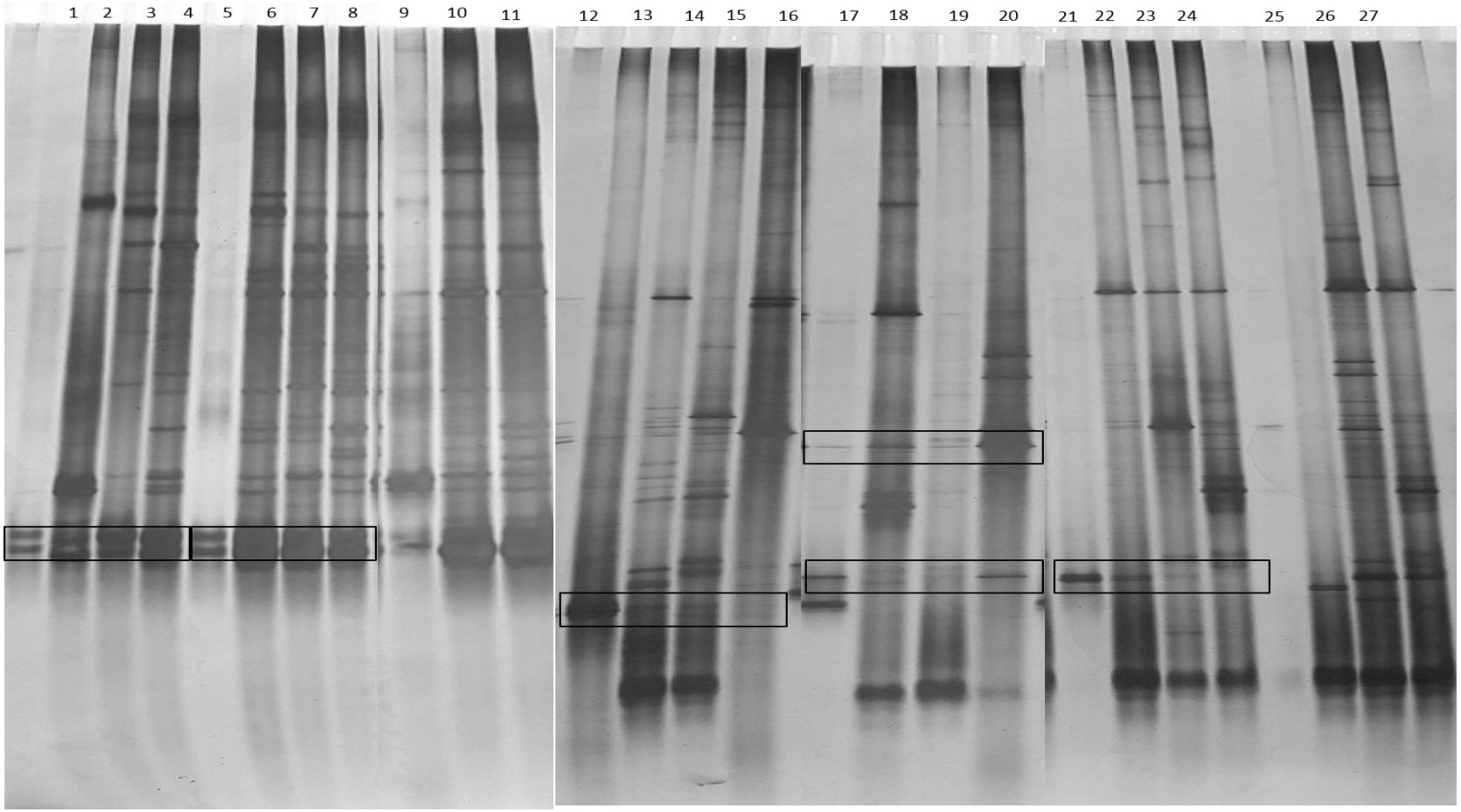


Figure 5.8: DGGE band patterns of pure cultures of endophytic Actinobacteria and fungi strains used as markers and DGGE band patterns of endophytic Actinobacteria (Lanes 1-11) and fungal communities (Lanes 12-27) obtained from roots of *P. colorata* seedling treated with the respective endophytes. Lane 1- *Nocardia* sp. TP1BA1B; Lanes 2-4 treatment TP1BA1B; Lane 5- *Streptomyces* sp. UKCW/B; Lanes 6-8 treatment UKCW/B; Lanes 9-12 Control seedlings; Lane 12- blank Lane 13- *Metarhizium* sp. PR1SB1; Lanes 14-16 treatment PR1SB1; Lane 17- *Xylaria* sp. P4LA3; Lane 18-20 treatment P4LA3; Lane 21- *Fusarium* sp. P4LC2; Lanes 22-24 treatment P4LC2; Lanes 25-27 Control seedlings.

5.3.3 Potential of endophytic fungi to biotransformation Farnesol

Addition of farnesol to the cultures of endophytic fungi did not increase or decrease the activity of filtrate from *Pezicula* sp. PRY2BA2 against *C. albicans*, but decreased the activity of the filtrate of *Fusarium* sp. P4LC2. The other endophytes used in the study lost activity after addition of farnesol. Addition of macerated leaf material to the flasks did not increase their activity against *C. albicans*. Plating the mycelium from the flask resulted in growth on PDA plates indicating that the cultures were viable but had lost activity after addition of farnesol and the leaf material.

5.4 Discussion

This study is the first to demonstrate that reinoculation of roots with endophytes isolated from *P. colorata* can alter the growth of *P. colorata* seedlings. This included fungal, Actinobacterial and bacterial endophytes and provided the first evidence for a role of these micro-organisms in growth of *P. colorata*. Results from Chapters 2 and 3 showed that endophytes isolated from *P. colorata* had the potential to protect against phytopathogens and to improve plant growth through nutrient mobilisation, however the mechanism by which growth promotion occurred was not explored.

Treating *P. colorata* seedlings with endophytic fungi resulted in increased plant heights and number of internodes for six treatments and five treatments, respectively, in comparison to the control. These results were consistent with those of Khan *et al*. (2016) where inoculation of the root zone of 4 week saplings of *Boswellia sacra* with the endophytic fungus *Preussia* sp. BSL 10 increased shoot length and internodes compared to the untreated control. Another study by Khan *et al*. (2012) showed that the endophytic fungus *Paecilomyces formosus* LHL10 increased the shoot length of cucumber seedlings by 6.89% compared to the untreated control. A study by Chirino-Valle *et al*. (2016) showed that the shoots lengths of *Miscanthus x giganteus* treated with an endophytic *Trichoderma* sp. were longer than the controls but were not different in terms of root and shoot biomass. These results were consistent with this study where the seedling treated with *Trichoderma* spp. increased the height of *P. colorata* seedlings but had no effect on the root and shoot dry weight. Endophytic fungi are known to increase plant growth due to the production of growth hormones (Schulz and Boyle, 2005) or by transporting nutrients to the host by organic matter mineralization (Newsham, 2011).

Auxins which are important plant growth hormones are known to be produced by some fungal species such as *Trichoderma* (Contreras-Cornejo *et al*., 2009; Mazhabi *et al*., 2011). However in this study, the exact mechanisms responsible for the increase in plant growth were not studied. Though the seedlings used in this study were not from the same seed lot, the endophytes had an effect on the plant growth irrespective of the host genotype, indicating that these endophytes are generalists.

In this study, *Bacillus* sp. TP1LA1B, in addition to increasing the shoot height, also significantly increased the root, shoot biomass and the number of internodes of *P. colorata* seedling compared to the control. These results are in accordance to the study by Zhao *et al*. (2015), which showed that a *Bacillus* sp. isolated from the medicinal plant *Lonicera Japonica* was able to increase the shoot length, root length as well as the fresh weight and dry weight of wheat seeds. A study by Quecine et *al.* (2012) demonstrated that the endophytic isolate *Pantoea agglomerans* 33.1 increased the length and biomass of only the aerial tissue of sugar cane seedlings but not the mass of roots. Another study by da Silva *et al*. (2015) demonstrated that *Pantoea ananatis* SCB4789F-4 isolated from sugarcane leaves promoted the shoot growth of 7 d old *Arabidopsis thaliana* seedlings. In this study *Pantoea* sp. AP1SA1 increased the shoot length of *P. colorata* seedling but did not increase the root and shoot dry weight. Although several studies have shown the ability of *Pantoea* sp. to stimulate plant growth, the exact mechanism has been considered as a complex phenomenon and needs further research (Kang *et al*., 2007; da Silva et al., 2015).

Phosphate solubilizing microbes are able to convert insoluble phosphorus to a soluble form thereby presenting a possible mechanism of direct plant growth under field conditions (Verma *et al.,* 2001). Although in the TCP plate assay (Chapter 4), *Streptomyces* sp. UKCW/B formed bigger clearance zones compared to *Nocardia* sp. TP1BA1B, in the *in planta* study *Nocardia* sp. TP1BA1B performed better by increasing both root and shoot biomass compared to the control and *Streptomyces* sp. UKCW/B. This suggests that *Nocardia* sp. TP1BA1B influences *P. colorata* growth via a different mechanism, which has not been revealed by the *in vitro* analyses in this study. Bashan *et al*. (2013a) showed that inoculating plants with endophytes that can solubilize phosphate *in vitro* may aid in plant growth but these results were highly variable. Fernandez *et al*. (2007) inoculated soybean with 13 isolates that solubilized TCP *in vitro* but under greenhouse conditions none of the isolates increased

growth. Taurian *et al*. (2010) demonstrated that of 110 bacteria that solubilized TCP only 1 strain increased the growth of peanut *in vivo*. Thus, the results of this study in combination with Chapter 4 are consistent with these published accounts and only 1 of the 2 strains of TCP solubilizing endophytic Actinobacteria increased the shoot and root dry of *P. colorata* seedlings. Mean shoot length (n=10) of the seedlings treated with the endophytic Actinobacteria *Nocardia* sp. TP1BA1B and *Streptomyces* sp. UKCW/B were 1.7 × and 1.6 × longer compared to untreated controls. These results were similar to the findings of Verma *et al*. (2011), where the shoots of the tomato seeds treated with a spore suspension of an endophytic *Streptomyces* AzR-051 were significantly longer than the untreated control. However, to understand the mechanism by which these endophytic Actinobacteria enhance plant growth, additional studies under different conditions such as phosphate deficient soils, potential to survive in soil and rhizosphere colonization potential are required.

This study acknowledges that the endophytic colonization was not confirmed by re-isolation for any of the endophytic inoculants applied in this study. However pure cultures of the Actinobacteria and fungal inoculants were run as reference markers in DGGE gels along with the treatments and compared to uninoculated control to check for the presence or absence of the marker bands. The marker bands of the endophytic Actinobacteria *Nocardia* sp. TP1BA1B and *Streptomyces* sp. UKCW/B used in this study were detected in their respective treatments and were absent in the control lanes indicating that the strains were able to re-colonize the roots of *P. colorata* seedlings. Out of seven endophytic fungi used in this study, marker bands of only three isolates were identified in their respective treatments. Bands corresponding to *Metarhizium* sp. PR1SB1, *Xylaria* sp. P4LA3 and *Fusarium* sp. P4LC2 were detected in the respective treatments. While the reference markers of some endophytes used in this study were not detected in the treatment gels in DGGE, they still had an effect on the growth of *P. colorata* seedlings. The bands of the endophytic *Trichoderma* sp. PRY2BA21, *Trichoderma* sp. PRY3BC1, *Chaetomium* sp. PR1BC2 used in this study were not detected in their respective treatments, yet they increased the shoot length of *P. colorata* seedlings. These findings indicate that the effect in these treatments could be due to a nutrient flush, especially as these endophytes are also commonly known to also exist as free living saprophytes or associated with the rhizosphere. For future studies, to clearly understand if the effect on fungi on growth of the plant is a result of nutrient flush, using nutrient deficient

soils in addition to potting mix could be beneficial. *Trichoderma* sp. are known for their ability to colonize the rhizosphere. Study by Cripps-Guazzone (2014) showed that different plants had different receptiveness to colonization by *Trichoderma* sp. where rye grass and cauliflower were more receptive to colonization compared to other plants such as onion, sweet corn, carrot and clover. So in this study, it is possible that the *Trichoderma* sp. were able to colonize the rhizosphere but could not become endophytic. However additional work in the future such as assessing the rhizosphere competence of isolates will be useful to understand the mechanism of growth promotion in this case.

Addition of some inoculants had an influence on the richness of Actinobacteria communities in the roots of *P. colorata* seedlings. Though in this study, five soil applied endophytes colonized *P. colorata* roots, they had no influence on the endophyte communities in the roots, with the exception of *Metarhizium* sp. PR1SB1 which reduced the richness of Actinobacteria. However, for future studies, additional tissues (leaves and stems) could be analysed to understand the breadth of colonization and to give insights into the mechanism that influences the plant growth and microbial communities. *Bacillus* sp. TP1LA1B increased the richness of Actinobacteria in the roots of *P. colorata* seedlings. Addition of endophytic Actinobacteria *Nocardia* sp. TP1BA1B and *Streptomyces* sp. UKCW/B did not influence the endophytic Actinobacteria communities in the roots of *P. colorata*. These results are consistent with Conn and Franco, (2004) where treatment of wheat with endophytic *Streptomyces* sp. strain EN27, *Microbispora* sp. strain EN2 and *Nocardiodes albus* EN46 did not influence the indigenous endophytic Actinobacteria in wheat seedlings. Though *Trichoderma* sp. PRY3BC1 was not shown to colonize the roots of *P. colorata* seedlings, it increased the richness of endophytic fungi in the roots of *P. colorata* in addition to increasing the shoot length. This could be a result of nutrient flush, which in turn influenced the recruitment of fungi by *P. colorata*. This however has not been tested, but for future studies, analysing the microbial communities of the soil/potting mix would be helpful to better compare the changes in the microbial community profile. This is the first study to report the influence of inoculation with endophytic Actinobacteria, bacteria and fungi strains on the endophytic communities in the roots of *P. colorata*.

The production of polygodial in *P. colorata* is likely to be a complex process (Pers. Comm. Nigel Perry). This study did not demonstrate any relationship between the endophytes and

production of polygodial *in vitro*. Although there are no published studies showing production of polygodial from a microbial source there is evidence that other plants such as *Drimys winteri* (Winteraceae), *Polygonum punctatum* (Polygonaceae) and *Warburgia ugandensis* Sprague (Canellaceae) also produce polygodial but the involvement of micro-organisms cannot necessarily be discounted. Ideally it would have been useful to measure if there were changes in polygodial levels in the endophyte inoculated seedlings. However, due to the seedlings being from different seed lots, there was very high variability in the levels of polygodial between plants as determined by NMR (Appendix D.5). For future studies, using seedlings from the same seed lot will be useful to reduce the levels of variability in the total sesquiterpene content. In addition, because *P. colorata* is an extremely slow growing plant, future growth trials should be carried out for longer than 4 months to get a better understanding of the differences in root and shoot weights.

In conclusion, for the first time this chapter demonstrates a role for micro-organisms in the growth of *P. colorata*. The results also showed that reinoculation with endophytes could alter microbial communities in the roots. None of the endophytes produced polygodial *in vitro,* even in the presence of a precursor compound and/or plant material. However, this has not discounted a role in metabolite production. The study did not investigate the effect of micro-organisms on polygodial production *in planta* but given the observed growth effects that is an area that warrants further investigation in the future.

Chapter 6

Concluding Discussion

The aim of this thesis was to analyse the community structure and functional properties of the endophytic bacteria, Actinobacteria and fungi inhabiting *Pseudowintera colorata* (horopito) using culturable and non-culturable approaches. There was particular focus placed on examining whether the cultured endophytes could inhibit phytopathogens and influence plant growth or chemistry. This is the first study on the endophytes inhabiting *P. colorata* and contributes to reducing the knowledge gap that exists in the realm of microbial associations with native plants. The only other comprehensive study on endophytic associations with a New Zealand native plant was by Wicaksono *et al*. (2016) on *Leptospermum scoparium* (mānuka) and revealed a key role for endophytic bacteria in the growth and essential oil chemistry of that plant.

Pseudowintera colorata is recognized as a medicinal plant due to the antimicrobial properties of its compounds, especially polygodial in the leaves. It has been used by māori in traditional medicine and, thus, holds an important position in ethnobotany in New Zealand. With international studies showing that medicinal plants harbour diverse endophyte communities, the traditional use of the plant and combined with the knowledge from the study on *L. scoparium*, the possibility of *P. colorata* harbouring unique endophytes with a role in plant growth and/or chemistry needed to be explored.

The structure of the endophytic bacteria, Actinobacteria and fungal communities in *P. colorata* was investigated in Chapter 2 using two different molecular approaches, DGGE and metabarcoding with the Illumina MiSeq platform. Using DGGE and Illumina MiSeq it was revealed that tissue type was the main factor influencing the composition and richness of bacteria, Actinobacteria and fungal endophytes. These findings were consistent both with the international literature and the study on *L. scoparium* by Wicaksono *et al*. (2016). Though tissue maturity was analysed only for a subset of three sites, the results indicated that tissue maturity influenced the community structure of all groups analysed except Alphaproteobacteria. However, the maturity of the plant did not have any effect on the richness across all groups. The clustering of Betaproteobacteria and fungi in leaves of immature plants but not in leaves of mature plants suggested a community shift for these

groups. For future studies, additional sites with adjacent immature and mature plants need to be sampled to get a conclusive understanding of the effect of plant maturity and location on endophyte communities. In addition, this study did not analyse the endophytic communities in the seeds of *P. colorata*. Studies have demonstrated that endophytes associated with seeds may be beneficial to the hosts (Wang *et al*., 2016). So future studies, should include analysis of seeds from plants of different maturity. This would be an important aspect to explore as the seeds and leaves of *P. colorata* are the tissues known to produce polygodial, and understanding the changes in communities might provide additional insights into the role of endophytic communities in plant chemistry. Sequencing the major bands from Actinobacteria DGGE gels revealed that some of them belonged to uncultured bacteria and may have been novel Actinobacteria. However, using DGGE for this study highlighted the technique's drawback that in order to determine what the identity of the other bands was, the only option was to sequence all the bands, which is both time consuming and at times difficult depending on how close the bands are. This was overcome to some degree by using Illumina MiSeq, which gave better identification and resolution.

Based on the DGGE analysis and the previous work of Wicaksono *et al*. (2016), which revealed immature *L. scoparium* plants were highly variable, only mature plants were considered for Illumina MiSeq analysis. While the sequencing of 16S rRNA region worked, Illumina MiSeq could not resolve the amplicons for the ITS2 region with more than 98% of the reads not passing quality filtering in QIIME. The same result was obtained when primers to amplify the ITS1 region were used. Data from DGGE and metabarcoding with Illumina MiSeq analysis for the endophytic bacteria were complementary to one another and this was consistent with other studies in the literature (Li *et al*., 2016b; Qin *et al*., 2016, Wicaksono, 2016). A greater level of identification, detection of microorganisms and taxonomic resolution was achieved by Illumina MiSeq compared to sequencing the excised DGGE bands. The data revealed that 89.1% of the total reads belonged to class Gammaproteobacteria. In addition, there was evidence of the presence of a core bacterial endomicrobiome in *P. colorata*. Though the definition of core endomicrobiome has been variable in the literature, the basic definition is the number of OTUs present in at least 50% of the samples. A study by Cardinale *et al*. (2015) on lettuce revealed 68 OTUs representing 49% of the total reads as a part of the lettuce root microbiome. The study of Sánchez-López *et al*. (2017) revealed *Methylobacterium* as the

dominant OTU and constituted more than 80% of the core microbiome in the seeds of *Crotalaria pumila*. In the present study, two OTUs belonging to the genus *Pseudomonas* were present in 75% of the samples and were identified as members of the core endomicrobiome of *P. colorata*. This is the first study to identify a core endomicrobiome in *P. colorata*. These findings were consistent with other studies which revealed the presence of a core endomicrobiome in other plants such as *Leptospermum scoparium* (Wicaksono, 2016), *Crotalaria pumila* (Sánchez-López *et al*., 2017) and *Cannabis sativa* (Winston *et al*., 2014). Future studies should investigate the importance of these OTUs in *P. colorata*, with specific attention to the possible roles in plant metabolism and growth. If they can be cultured, re-inoculation into endophyte free *P. colorata* could reveal potential effects on growth, metabolism and/or physiology.

Though a greater level of information was available through Illumina MiSeq, the choice of primers affected the overall output of data in this study. Both the primers for 16S and ITS2 regions amplified chloroplast, plastid and mitochondrial DNA. Chloroplasts are commonly amplified during PCR for DGGE (Bulgarelli *et al*., 2012; Dorn-In *et al*., 2015), but the use of group specific primers for DGGE mitigated the problem to an extent. However, with Illumina MiSeq the primers used were universal for the V3-V4 hypervariable region of the 16S rRNA region and post analysis 98.9% of reads were removed as assigned to chloroplast DNA based on both Greengenes (data in this study) and NCBI database (data not shown). With a large amount of data being removed, this study is unlikely to represent fully the communities of endophytic bacteria in *P. colorata*. Future studies, should use primers that would reduce the amplification of non-ribosomal DNA and plant DNA for example, 16S rRNA primers such as 799F and 1391R (Beckers *et al.,* 2016). In this study, prior to extracting DNA, surface sterilized tissues were treated with PMA, which binds to DNA from extraneous and non-viable cells which still remain after surface sterilization (Carini *et al*., 2016, Wicaksono, 2016). This was valuable to enrich for true endophyte DNA and future studies describing the communities of endophytes should incorporate PMA treatment of tissues prior to extracting DNA. In addition, the method of extracting DNA for such studies is just as critical as the choice of primers. For future studies, the outcome of sequencing could be improved by using refined extraction methods that can specifically exclude plant (chloroplast and mitochondria) DNA (Lutz *et al.,*

2011) and separating bacterial and plant cells by usage of density gradient centrifugation (Chapelle *et al*., 2015).

Along with metabarcoding with improved primers, using metagenomics for future studies could reveal the role of endophytes in the productions of novel secondary metabolites and enzymes in addition to contribution to the physiology and metabolism of *P. colorata*. Study by Müller *et al*. (2015b) on *Sphagnum* Bog metagenome found 13 novel enzymes belonging to NRPSs (nonribosomal peptide synthetases) from the members of phyla Proteobacteria, Actinobacteria and Cyanobacteria. Yuan *et al*. (2016), found that the endophytic bacteria in the microbiome of halotolerant plant *Suaeda salsa* (seepweed) had functional gene categories related to salt stress acclimatization, nutrient solubilisation and competitive root colonization.

The isolation and bioactive potential of the culturable endophytic bacteria and fungi from *P. colorata* was demonstrated in chapter 3. Several international studies reported that roots host a higher number of endophytes, compared to stems and leaves (Jin *et al*., 2014; Wicaksono *et al*., 2016). In this study, the number of endophytic bacteria and fungi isolated from the stems of *P. colorata* was higher than the roots and leaves with the leaves yielding the lowest number of culturable endophytic bacteria (n=20) and no culturable Actinobacteria. Several endophytes isolated from *P. colorata* were strongly inhibitory against phytopathogenic fungi *Neofusicoccum luteum*, *N. parvum*, *Neonectria ditissima*, *Ilyonectria liriodendri*, and phytopathogenic bacteria *Pectobacterium atrosepticum* and *P. brasiliensis in vitro*. Several international studies have reported the potential of endophytes from medicinal plants as biocontrol agents (Miller *et al*., 2012; Tianxing *et al*., 2013). The selection of these pathogens was based on their recognition as aggressive pathogens in New Zealand and globally, with limited success from chemical fungicides and bactericidal agents (Gnanamanickam and Charkowski, 2006; Augustí-Brisach and Armengol, 2013). Though plate based assays revealed the activity of endophytes against phytopathogens, future studies will need to determine the ability of the endophytes to control phytopathogens *in vivo* and especially using *P. colorata* as a model to identify the potential role of endophytes in protecting the host. For example, Wicaksono *et al*. (2017) demonstrated that the endophytic bacteria recovered from *L. scoparium* active against *Pseudomonas syringae* pv. *Actinidiae in*

vitro were also able to inhibit the colonization of pathogen and reduced the disease severity in kiwi fruit.

In this study, the inhibition of phytopathogens was likely by the production of antibiotics. Using PCR to detect the antibiotic genes it was revealed that one *Pseudomonas* sp. produced a known antibiotic phenazine. It is likely that the other *Pseudomonas* sp. in this study could be producing other antibiotics that were not detected or even new antibiotics. Future studies would need to look at these potential compounds and mechanisms by extracting the pure compounds from cell free culture filtrates and testing the activity against phytopathogens.

The leaves of *P. colorata* are known to produce polygodial, which is used to treat candidiasis and Māori used the leaves to treat skin infections and gonorrhea. Kubo *et al*. (2001 and 2005) demonstrated that polygodial was active against bacterial pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and fungal pathogens such as *Candida albicans*. Parallels with other international studies demonstrated that some endophytes from medicinal plant produced the same compounds as the host for example, the endophytic fungi *Taxomyces andreanae* was capable of producing the anticancer drug, Taxol™ (Paclitaxel) similar to its host Pacific yew tree, *Taxus brevifolia* (Wani *et al.,* 1971). With *P. colorata* being identified as a medicinal plant, and evidence in ethnobotany for treating human infections, the potential of the endophytes against some opportunistic human pathogens needed to be explored. In addition to phytopathogens, the endophytic bacteria, Actinobacteria and fungi were also tested against opportunistic human pathogens *S. aureus*, *E. coli* and *C. albicans*. Studies have identified endophytic fungi for their potential in producing a wide variety of bioactive compounds including the same or similar compound produced by the host plant (Bezerra *et al.,* 2015). Several endophytic fungi isolated from *P. colorata* showed activity against *C. albicans* with clearance zones > 10mm.

Previous studies have reported endophytic Actinobacteria from medicinal plants as a source of novel compounds (Trujillo *et al*., 2015; Golinska *et al.,* 2015). In addition to producing bioactive compounds, endophytic Actinobacteria can improve plant growth and tolerance of adverse conditions (Hasegawa *et al*., 2006; Cao *et al*., 2005). Endophytic Actinobacteria are involved in turning over organic matter and promoting plant growth through assimilation of iron and other nutrients (Tokala *et al*., 2002; Coombs and Franco, 2003b). However, there are no published studies on endophytic Actinobacteria in a native New Zealand plant. DGGE

analysis in chapter 2 revealed that tissues of *P. colorata* harboured a community of Actinobacteria, with lanes showing an average of 13 bands from different tissues. However, the number of culturable endophytic Actinobacteria (n=9) isolated in Chapter 4 was comparatively low. In this study only two types of agar were used, while other international studies have employed 5-10 types of agar (Kaewkla and Franco, 2013; Passari *et al*., 2015), thus increasing the recovery of Actinobacteria from tissues. For future studies, the choice of media type and number of agar types would be a critical aspect to consider.

Using Illumina MiSeq in Chapter 2 revealed that only 1.2% of the total reads belonged to phylum Actinobacteria. In contrast the study by Pinto *et al.* (2014) revealed that Actinobacteria made up 19% of the microbial community composition in *Vitis vinifera*. As discussed earlier, in this study, the percentage of Actinobacteria could only be inferred as a representation because of the majority of the reads being removed as chloroplasts and mitochondria. For future studies, using the specific primers for NGS as used for DGGE might help in giving more information about the diversity of endophytic Actinobacteria in *P. colorata*.

Some of the culturable endophytic Actinobacteria demonstrated activity against the phytopathogenic fungi tested, produced siderophores and solubilized phosphate. While the function of Actinobacteria on the host plants is unknown for most of the plant studied so far (Trujillo *et al*., 2015), Cardinale *et al.* (2015) suggested that the presence of endophytic Actinobacteria such as *Streptomyces* in roots of lettuce may serve as biocontrol agents by producing antibiotics to eliminate phytopathogens. Endophytic Actinobacteria have gained attention for their potential as biocontrol agents due to their ability to colonize healthy plant tissues and produce antibiotics *in situ* (Cao *et al*., 2004). The potential role of endophytic Actinobacteria on *P. colorata* was unexplored and, based on their novelty, profile in the international literature and ability to solubilize phosphate, these strains were selected to study their effect on *P. colorata* growth in Chapter 5.

This is the first study to report the effects of inoculation of endophytes isolated from *P. colorata* on the growth of *P. colorata* seedlings. Combined results from Chapter 2 and 3 showed that in addition to protecting against phytopathogens, the endophytes from *P. colorata* had the potential to improve plant growth through nutrient mobilisation, however the mechanism by which growth promotion occurred was not explored. The re-colonization

of endophytes used in this study was confirmed by co-migration of pure cultures as reference markers in DGGE gels and comparing to untreated controls for the presence or absence of reference markers. Though this process confirmed the presence of the endophytes in the treated seedlings, colonization of seedlings as endophytes was not visualized. Future studies should use FISH/CLSM or other advanced microscopy techniques to visualize the colonization of endophytes. Of the endophytes used in this study (n=11), two endophytic Actinobacteria and three endophytic fungi were able to colonize the roots of *P. colorata* seedlings. From the endophytes used in this study, 10 treatments increased the growth of *P. colorata* seedlings compared to the untreated control. For example, mean shoot length (n=10) of seedlings treated with *Trichoderma* sp. and *Pantoea* sp. were 2.2 × and 1.8 × times longer than the untreated control. In addition to increasing length, some endophytes like *Nocardia* sp. and *Bacillus* sp. increased the dry weight of shoots (1.8 × and 1.5 ×, respectively) and roots (1.6 × and 1.5 ×, respectively) in addition to increasing the number of internodes (1.6 × and 1.8 ×, respectively). This was the first study to demonstrate the influence of endophytes on the growth of *P. colorata* and confirmed their role in the ecology of the host. However, the persistence of these endophytes or movement within tissues was not analysed in this study and needs to be done in future studies to understand the mechanism by which endophytes influence the host. A study by Wicaksono, (2016) demonstrated the colonization and persistence of endophytic bacteria in the tissues of grapevine. Some of the treatments which were not shown to colonize *P. colorata* roots were still able to increase the growth of *P. colorata* seedlings, which could have been due to nutrient flush. However, this was not tested in this study and future studies will need to use different types of soils including nutrient deficient soils to understand the differences in phenotypes of the host. In addition, the rhizosphere competence of some well know endophytic fungi like the *Trichoderma* sp. used in this study needs to be investigated in future to understand how they influenced the growth of *P. colorata*.

The inoculation of some of the endophytes influenced the microbial communities in the roots of the *P. colorata* seedlings irrespective of their colonization. The mechanism by which these endophytes influenced the communities in *P. colorata* roots remained unexplored and needs to be explored in future studies. International studies have suggested that this could be the effect caused by the inoculants, which outcompete the indigenous flora and thus influencing

the communities (Conn and Franco, 2004) or in some cases such as *Trichoderma* sp. which influence the microbial communities as a result of their rhizosphere competence. Using advanced microscopic techniques such as confocal microscopy the endophytic colonization in *P. colorata* can be visualized to fully understand the process.

This study could not demonstrate any relationship between the endophytes and production of polygodial by *in vitro* assays. Analysis of the culture filtrates using NMR did not show any peaks in the dialdehydes region corresponding to polygodial and 9-deoxymuzigadial. Since only cell free culture filtrates were used for analysis, future work would need to use fungal mycelia to identify if the compounds were sequestered in the mycelial mass. Prior to inoculation of endophytes, the total polygodial content of random leaves of the seedlings was measured using NMR and as the seedlings belonged to different seed lots, the variability in polygodial and 9-deoxymuzigadial was too high. Thus, post inoculation the changes in polygodial level were not measured. For future *in planta* studies, selection of seedlings from the same lot is critical to reduce the variability in levels of polygodial. Work by Wayman *et al*. 2010 and Perry *et al*. 1996(b) showed that *P. colorata* from four different locations were of varying chemotypes and differed in the levels of polygodial. In this study, though the plants were sampled at 10 different sites from North and South Island, the chemotype of the plants was not tested, which would have given an additional parameter along with tissue type, maturity, and location differences, to correlate with community structure and microbial diversity. Wayman *et al.* 2010 showed that the fruits of *P. colorata* contained high levels of polygodial and 9-deoxymuzigadial. In addition, as there is evidence that there is a community shift as the *P. colorata* matures, it would be interesting to know what original communities were associated with *P. colorata*. Thus, future work should focus also on the seeds of *P. colorata* from different sites (North and South Island) to fully understand the influence of chemotype on the diversity of vertically transmitted endophytes in *P. colorata*.

In summary, this study is the first exploration of the community structure, identity and function of microbial endophytes in *P. colorata*. *Pseudowintera colorata* is not only of economic importance to New Zealand but also has a great cultural significance. The results from this study showed that the microbes associated with *P. colorata* form tissue specific groups and are likely to be important to its ecology through an effect on growth. Cultured members of the community were also strongly antimicrobial *in vitro*, decreasing the growth

of plant and human pathogens. A small number of endophytic fungi (n=7) and bacteria (n=2) affected the growth of yeast, however NMR analysis of the culture filtrates revealed that the filtrates did not contain the sesquiterpene dialdehydes polygodial and 9-deoxymuzigadial. However the ability of the fungal mycelium to sequester these compounds was not confirmed. Future studies, will need to examine if the endophytic fungi sequester these compounds in liquid culture and compare the profiles to polygodial standard to identify if the endophytes produced polygodial or similar compounds. The work here has demonstrated several promising areas of future research including the mechanistic basis of the observed growth promotion, potential as biocontrol agents and the production of new compounds. The culture collection and basic understanding produced by this research provides a good platform for such future studies.

Conference presentations

Purushotham N., Jones E. E., Monk J., and Ridgway H. J. (2014) Endophytes of horopito (*Pseudowintera colorata*) and their bioactive properties. The New Zealand Microbiological Society conference, Wellington, New Zealand.

Purushotham N., Jones E. E., Monk J., and Ridgway H. J. (2015) Antimicrobial potential of bacterial endophytes that inhabit New Zealand's medicinal plant *Pseudowintera colorata*. New Zealand Plant Protection Society Conference, Christchurch, New Zealand.

Purushotham N., Jones E. E., Monk J., and Ridgway H. J. (2016) Analysing the endomicrobiome of New Zealand's medicinal plant *Pseudowintera colorata*. The International Society of Microbial Ecology Conference, Montreal, Canada.

Purushotham N., Jones E. E., Monk J., and Ridgway H. J. (2016) Analysing the bioactive potential of the endomicrobiome of New Zealand's medicinal plant *Pseudowintera colorata*. The International Organization of Biological Control- WPRS conference, Berlin, Germany.

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Appendix A

A.1 List of sampling sites of *P. colorata* for DGGE and Illumina MiSeq metabarcoding

Site Location	Latitude	Longitude	Region
Taihape Scenic Reserve	-39.67635	175.80560	Manawatu-Wanganui
Tongariro National Park	-39.02237	175.71810	Manawatu-Wanganui
Kaimanawa Forest Park	-38.94721	175.94370	Manawatu-Wanganui
Lake Rotopounamu Scenic Reserve	-39.02656	175.73502	Manawatu-Wanganui
Arthur's Pass National Park	-42.94215	171.56414	Canterbury
Kaituna Valley Scenic Reserve	-43.71655	172.7554	Canterbury
Peel Forest	-43.91835	171.25934	Canterbury
Paringa Forest	-43.69379	169.40724	West Coast
Otago Peninsula Scenic Reserve	-45.88184	170.58049	Otago
Kahurangi National Park	-41.07224	172.59166	Nelson/Tasman

The permission to sample the sites was obtained from DOC dated 25/09/2014 with the

National Authorisation Number: 39368-FLO

A.2 CTAB Buffer

Reagent	/250 mL
Hexadecyltrimethyl ammonium bromide (CTAB)	5 g
5 M NaCl	70 mL
0.5 M EDTA (pH 8.0)	10 mL
1 M Tris-Cl (pH 8.0)	25 mL
Polyvinylpyrrolidone (PVP) (MW 40 kDa)	2% (w/v)
β-Mercaptoethanol	1% (v/v)
H_2O	145 mL

A.3 TAE Buffer (50X)

1M Tris (hydroxymethyl) aminomethane (Sigma Aldrich	242 gm
0.5 M Sodium EDTA	100 mL
Glacial Acetic Acid (Labserv)	57.1 mL
RO water	1000 mL
for 1x TAE buffer: 40 mL 50x buffer was added to 1960 mL RO water.	

A.4 Ethidium Bromide (EtBr)

25 μL of stock EtBr (10 mg/mL) dissolved in 500 mL of RO water was used for staining

A.5 Concentration of DNA used for Illumina MiSeq sequencing for the different samples

Site	Site code	Tissue	Concentration ng/mL
Arthur's Pass	AP	Leaf	13.5
		Stem	15.2
		Root	16.5
Kiko Road	KI	Leaf	21.0
		Stem	15.9
		Root	13.3
Tongariro Nat. Park	TO	Leaf	12.1
		Stem	12.4
		Root	12.7
Taihape Reserve	TP	Leaf	12.4
		Root	12.6
		Stem	8.14
Lake Rotopounamu	RO	Stem	11.1
		Stem	8.05
		Leaf	7.38
Otago	OT	Leaf	19.1
		Stem	12.1
Kahurangi Nat. Park	KH	Leaf	12.4
Peel Forest	PL	Leaf	15.8
		Stem	7.13
		Root	18.4
Paringa	PR	Leaf	12.8
		Stem	8.06
		Root	15.4
	PRY 1	Leaf	12.6
	PRY 2	Leaf	26.3
	PRY 3	Leaf	35.3
Kaituna Valley	KV	Leaf	27.8
		Stem	22.9
		Root	14.0
	KVY	Leaf	35.6

A.6 Sequence of the 16S rRNA primers including Illumina flow cell adaptors and unique barcode used in the study

Primer code	Primer Sequence (5' to 3')
341F_SC501	AATGATACGGCGACCACCGAGATCTACACACGACGTGTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SC502	AATGATACGGCGACCACCGAGATCTACACATATACACTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SC503	AATGATACGGCGACCACCGAGATCTACACCGTCGCTATATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SC504	AATGATACGGCGACCACCGAGATCTACACCTAGAGCTTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SC505	AATGATACGGCGACCACCGAGATCTACACGCTCTAGTTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SC506	AATGATACGGCGACCACCGAGATCTACACGACACTGATATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SC507	AATGATACGGCGACCACCGAGATCTACACTGCGTACGTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SC508	AATGATACGGCGACCACCGAGATCTACACTAGTGTAGTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SD501	AATGATACGGCGACCACCGAGATCTACACAAGCAGCATATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SD502	AATGATACGGCGACCACCGAGATCTACACACGCGTGATATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SD503	AATGATACGGCGACCACCGAGATCTACACCGATCTACTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SD504	AATGATACGGCGACCACCGAGATCTACACTGCGTCACTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SD505	AATGATACGGCGACCACCGAGATCTACACGTCTAGTGTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SD506	AATGATACGGCGACCACCGAGATCTACACCTAGTATGTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SD507	AATGATACGGCGACCACCGAGATCTACACGATAGCGTTATGGTAATTGGCCTACGGGNGGCWGCAG
341F_SD508	AATGATACGGCGACCACCGAGATCTACACTCTACACTTATGGTAATTGGCCTACGGGNGGCWGCAG
805R_SC701	CAAGCAGAAGACGGCATACGAGATACCTACTGAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC702	CAAGCAGAAGACGGCATACGAGATAGCGCTATAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC703	CAAGCAGAAGACGGCATACGAGATAGTCTAGAAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC704	CAAGCAGAAGACGGCATACGAGATCATGAGGAAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC705	CAAGCAGAAGACGGCATACGAGATCTAGCTCGAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC706	CAAGCAGAAGACGGCATACGAGATCTCTAGAGAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC707	CAAGCAGAAGACGGCATACGAGATGAGCTCATAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC708	CAAGCAGAAGACGGCATACGAGATGGTATGCTAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC709	CAAGCAGAAGACGGCATACGAGATGTATGACGAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC710	CAAGCAGAAGACGGCATACGAGATTAGACTGAAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC711	CAAGCAGAAGACGGCATACGAGATTCACGATGAGTCAGTCAGCCGACTACHVGGGTATCTAATCC
805R_SC712	CAAGCAGAAGACGGCATACGAGATTCGAGCTCAGTCAGTCAGCCGACTACHVGGGTATCTAATCC

A.7 Sequence of the ITS2 primers including Illumina flow cell adaptors and unique barcode used in the study

Primer code	Primer Sequence (5' to 3')
ITS4_SC701	CAAGCAGAAGACGGCATACGAGAT ACCTACTG AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC702	CAAGCAGAAGACGGCATACGAGAT AGCGCTAT AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC703	CAAGCAGAAGACGGCATACGAGAT AGTCTAGA AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC704	CAAGCAGAAGACGGCATACGAGAT CATGAGGA AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC705	CAAGCAGAAGACGGCATACGAGAT CTAGCTCG AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC706	CAAGCAGAAGACGGCATACGAGAT CTCTAGAG AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC707	CAAGCAGAAGACGGCATACGAGAT GAGCTCAT AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC708	CAAGCAGAAGACGGCATACGAGAT GGTATGCT AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC709	CAAGCAGAAGACGGCATACGAGAT GTATGACG AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC710	CAAGCAGAAGACGGCATACGAGAT TAGACTGA AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC711	CAAGCAGAAGACGGCATACGAGAT TCACGATG AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
ITS4_SC712	CAAGCAGAAGACGGCATACGAGAT TCGAGCTC AGTCAGTCAG CC TCCTCCGCTTATTGATATGC
fITS7_SC501	AATGATACGGCGACCACCGAGATCTACAC CCTCTCTT TATGGTAATT GG GTGARTCATCGAATCTTTG
fITS7_SC502	AATGATACGGCGACCACCGAGATCTACAC ATATACAC TATGGTAATT GG GTGARTCATCGAATCTTTG
fITS7_SC503	AATGATACGGCGACCACCGAGATCTACAC TATGGCAC TATGGTAATT GG GTGARTCATCGAATCTTTG
fITS7_SC504	AATGATACGGCGACCACCGAGATCTACAC GTCCTTCG TATGGTAATT GG GTGARTCATCGAATCTTTG
fITS7_SC505	AATGATACGGCGACCACCGAGATCTACAC GCTCTAGT TATGGTAATT GG GTGARTCATCGAATCTTTG
fITS7_SC506	AATGATACGGCGACCACCGAGATCTACAC GACACTGA TATGGTAATT GG GTGARTCATCGAATCTTTG
fITS7_SC507	AATGATACGGCGACCACCGAGATCTACAC AACTCCGC TATGGTAATT GG GTGARTCATCGAATCTTTG
fITS7_SC508	AATGATACGGCGACCACCGAGATCTACAC AGGACATT TATGGTAATT GG GTGARTCATCGAATCTTTG
ITS2_Read 1 Primer	TATGGTAATTGGGTGARTCATCGAATCTTTG
ITS2_Read 2 Primer	AGTCAGTCAGCCTCCTCCGCTTATTGATATGC
ITS2_Index Read Primer	GCATATCAATAAGCGGAGGAGGCTGACTGACT NOTE: reverse compliment

A.8 Script used in QIIME 1.8.1 for Illumina MiSeq data analysis

1. Join paired end with fastq join

join_paired_ends.py -f forward_reads.fastq -r reverse_reads.fastq -o joined.fastq

2. Quality (Phred 15 and maxee 0.5) and length (400 bp) trimming of fastq file

-fastq_filter joined.fastq -fastq_trunclen 400 -fastq_truncqual 15 -fastq_maxee 0.5 -fastqout filtered.fastq

3. Combined fastq data from two Illumina Miseq runs

filteredrun1.fastq filteredrun2.fastq >filteredjoin.fastq

4. Converting to fasta from fastq file

fastq_to_fasta -i filtered.fastq -o filteredjoin.fasta

5. Combine all fasta file and add label according mapping file

add_qiime_labels.py -i Allfastafile -m mappingfile.txt -c combined_seqs.fasta

6. Pick OTUs, assign taxonomy, and create an OTU table against a reference set of OTUs

pick_closed_reference_otus.py -i combined_seqs.fasta -o otus/

7. Remove OTU belonged to chloroplast and Mitochondria from OTU table

filter_taxa_from_otu_table.py -i otu.biom -o otunonplant.biom -n c__Chloroplast,f__mitochondria

8. Run alpha and beta diversity analysis

core_diversity_analyses.py -o alldataset/ -i otunonplant.biom -m mappingfile.txt -t rep_tree.tre -e 448

9. Identify the core OTUs in otu_table.biom, defined as the OTUs that are present in at least 80% of the samples.

compute_core_microbiome.py -i otunonplant.biom -otu_table_core.biom

A.9 Details of the OTU reads assigned to chloroplast and mitochondria based on the Greengenes database

#OTU ID	Identity	Number of Sequences
4191382	chloroplast	2946
769222	chloroplast	109
4332202	chloroplast	48
1131894	chloroplast	153837
1141758	chloroplast	185
4282801	chloroplast	271
4302241	chloroplast	507
735769	chloroplast	1209618
432284	chloroplast	1516
3359884	chloroplast	949
2307137	chloroplast	329
192539	chloroplast	409
467605	chloroplast	185756
1787644	chloroplast	25992
1646255	mitochondria	3
1646259	mitochondria	3
1892252	mitochondria	3
4420570	chloroplast	460
1126072	chloroplast	244
1793401	chloroplast	407

Appendix B

B.1 Fungal Preservation Media (Dr Jana Monk as per comms.)	per 400 mL
Glycerol (LabServ, Thermofisher Scientific)	240 mL
Glucose (Scharlau, Scharlab S.L)	40 g/ 80 mL
Bacteriological Peptone (Difco, BD Company)	8 g/ 40 mL
Yeast Extract (Difco, BD Company)	4 g/ 40 mL

B.2 Waksman Agar (Opelt and Berg, 2004)

	per litre
Beef Extract (Acumedia, Neogen)	5 g
Bacteriological Peptone (Difco, BD Company)	5 g
Sodium Chloride (LabServ, Thermofisher Scientific)	5 g
Glucose (Scharlau, Scharlab S.L)	10 g
Agar (Difco)	15 g

pH adjusted to 7.2 before autoclaving

B.3 CAS agar (Schwyn and Neilands, 1987)

Blue Dye:

a. Solution 1: 0.06 g of CAS (Sigma-Aldrich Co. LLC) in 50 mL of ddH_2O.
b. Solution 2: 0.0027 g of $FeCl_3$-6 H_2O (LabServ, ThermoFisher Scientific Inc.) in 10 ml of 10 mM HCl.
c. Solution 3: 0.073 g of HDTMA (Sigma-Aldrich Co. LLC) in 40 mL of ddH_2O.
d. Solution 1 was mixed with 9 mL of Solution 2. The resulting solution was mixed with Solution 3. The final solution was blue colour. This solution/dye was autoclaved and store in a plastic container/bottle.

Mixture solution:

a. Minimal Media 9 (MM9) Salt Solution Stock

   15 g KH_2PO_4 (Sigma-Aldrich Co. LLC), 25 g NaCl (LabServ, ThermoFisher Scientific Inc.), and 50 g NH_4Cl (LabServ, ThermoFisher Scientific Inc.) was dissolved in 500 mL of ddH_2O.

b. 20% Glucose Stock

20 g glucose (Scharlau, Scharlab S.L) was dissolved in 100 mL of ddH_2O.

c. NaOH Stock

   Dissolve 25 g of NaOH (LabServ) in 150 mL ddH_2O; pH should be ~12.

d. Casamino Acid Solution

   3 g of Casamino acid (Sigma-Aldrich Co. LLC) was dissolved in 27 mL of ddH_2O. Extract with 3% 8-hydroxyquinoline in chloroform to remove any trace iron and filter sterilize.

CAS agar Preparation:

a. 100 mL of MM9 (Sigma-Aldrich Co. LLC) salt solution to 750 mL of ddH_2O.
b. 32.24 g piperazine-N,N'-bis(2-ethanesulfonic acid) PIPES (Sigma-Aldrich Co. LLC). PIPES was dissolved below pH of 5. pH was brought up to 6 and PIPES was slowly added while stirring. While stirring, the pH was brought up to 6.8, taking care not to exceed 6.8 as this will turn the solution green.
c. 15 g agar (Difco).
d. Autoclaved and cooled to 50^{o}C.
e. 30 mL of sterile Casamino acid solution and 10 mL of sterile 20% glucose solution was added to MM9/PIPES mixture.
f. 100 mL of Blue Dye solution was slowly added along the glass wall with enough agitation to mix thoroughly and without forming bubbles.

B.4 Waksman Broth

	per litre
Beef Extract (Acumedia, Neogen)	5 g
Bacteriological Peptone (Difco, BD Company)	5 g
Sodium Chloride (LabServ, Thermofisher Scientific)	5 g
Glucose (Scharlau, Scharlab S.L)	10 g

pH adjusted to 7.2 before autoclaving

B.5 Activity of endophytic bacteria against phytopathogenic fungi, bacteria, opportunistic human pathogens and secretion of siderophores on CAS agar

Isolate	Tissue Isolated from	Site	*N. luteum*	*N. parvum*	*I. liriodendri*	*N. ditissima*	*P. atrosepticum*	*P. brasiliensis*	*S. aureus*	*E. coli*	CAS
KVP1RC1	Root	Kaituna Valley	+	-	-	-	-	-	-	-	-
KRP1SC2	Stem	Kahurangi Nat. Park	+	+++	+	+	-	-	-	-	+++
KVYPRA1	Root	Kaituna Valley	-	-	-	-	-	-	-	-	+
KVP1RA2	Root	Kaituna Valley	-	-	-	-	-	-	-	-	-
KVYPBC2	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	-
KVYPBC1	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	+
KVYPBC3	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	++
KVYPBB1	Stem	Kaituna Valley	+	-	-	-	-	-	-	-	-
KVYPRC1	Root	Kaituna Valley	-	-	-	-	-	-	-	-	-
KVYPSC1	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	-
KVP1RA1	Root	Kaituna Valley	-	-	-	-	-	-	-	-	+
KVP1RB1	Root	Kaituna Valley	-	-	-	-	-	-	-	-	-
KVP1BC1	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	-
KVP1RC22	Root	Kaituna Valley	+	+	-	+	-	-	-	-	+++
KVP1RC21	Root	Kaituna Valley	+	+	-	-	-	-	-	-	-
KVP1RB2	Root	Kaituna Valley	-	-	-	-	-	-	-	-	+
KVP1RC1	Root	Kaituna Valley	-	-	-	-	-	-	-	-	+
KVP1RB1A	Root	Kaituna Valley	-	-	-	-	-	-	-	-	+
KVP1BB1	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	
KVP1RC1	Root	Kaituna Valley	+	-	-	-	-	-	-	-	++
APYRB1	Root	Arthurs Pass	-	+	+	+	-	-	-	-	+++
APYBA2	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYRB3	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-

Table B5 continued

APYBC1	Stem	Arthurs Pass	+	+++	+	+	+	-	-	-	+++
APYSA2	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYBA1	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYBB3	Stem	Arthurs Pass	-	-	-	+	-	-	-	-	++
APYBB2	Stem	Arthurs Pass	-	+	+	+	-	-	-	-	-
APYRB4	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYRA2	Root	Arthurs Pass	-	-	+	+	-	-	-	-	-
APYRA2X	Root	Arthurs Pass		+	+	+	-	-	-	-	+++
KRP1SC1	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	-
KVYPBA1	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	-
KVYPBA2	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	+
KVYPBA2X	Stem	Kaituna Valley	+	-	-	-	-	-	-	-	+
KVYPRA3	Root	Kaituna Valley	-	-	-	-	-	-	-	-	++
KVYPRA22	Root	Kaituna Valley	-	-	-	-	-	-	-	-	-
KVYPRB1	Root	Kaituna Valley	+	-	-	-	-	-	-	-	+
ROP1SB1	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
TOP1RC1B	Root	Tongariro Nat. Park	-	-	+	+	-	-	-	-	++
TOP1RA3B	Root	Tongariro Nat. Park	-	-	+		-	-	-	-	-
ROP1RB4B	Root	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
ROP1RB3B	Root	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
ROP1RB2B	Root	Lake Rotopounamu	++	+++	+	+	-	-	-	-	++
TP1RA1B	Root	Taihape	-	-	+	+	-	-	-	-	-
TP2SB1R	Stem	Taihape	-	-	-	-	-	-	-	-	-
ROP1LB1	Leaf	Lake Rotopounamu	-	-	+		-	-	-	-	-
KIP2RB2R	Root	Kaimanawa Forest	-	++	+	+	-	-	-	-	+++
KVP1RA2B	Root	Kaituna Valley	-	-	-	-	-	-	-	-	-
KRP1BC3	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	-
KRP1BC2	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	-

Table B5 continued

KRP1BC1	Stem	Kahurangi Nat. Park	+++	+++	++	+	-	-	+	-	+++
KRP1SC1	Stem	Kahurangi Nat. Park	-	-	-	+	-	-	-	-	-
KRP1LB1	Leaf	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	-
KRP1BB1	Stem	Kahurangi Nat. Park	+++	+++	+	+	-	-	+	-	+++
KRP1BA2	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	
KRP1BA1	Stem	Kahurangi Nat. Park	+++	+++	+	+	-	-	++	-	+++
KRP1BB2	Stem	Kahurangi Nat. Park	-	-	-	+	-	-	-	-	-
KRP1BB3	Stem	Kahurangi Nat. Park	-	-	-	+	-	-	-	-	-
KVP1BC1	Stem	Kaituna Valley	+	+	+	+	-	-	-	-	+++
KRP1SC1A	Stem	Kahurangi Nat. Park	-	+++	+	+	-	-	+++	-	-
AP1SB1	Stem	Arthurs Pass	-	-	+	+	-	-	-	-	-
KRP1SC3	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	-
KRP1LB1F	Leaf	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	++
KIP2SB1B	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	+
KIP2BB2	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	++
KIP2LA2B	Leaf	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
KIP1BA1R	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
ROP2SB2	Stem	Lake Rotopounamu	-	+	-	-	-	-	-	-	-
KRP1SB1	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	++
TOYPRC2R	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
KIP1LA1B	Leaf	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
TP2RC1B	Root	Taihape	-	-	-	-	-	-	-	-	+
ROP1SA1B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	+
TOYPSC1R	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	+
KIP1RB2B	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	+
ROP2SC3B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	+
ROP2SC2B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	+
ROP2SC1B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	+

Table B5 continued

KIP2SA3R	Stem	Kaimanawa Forest	+	+	+	+	-	-	-	-	+
KIP2SA2R	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
TOYPLA1B	Leaf	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
KIP2SA1R	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
AP1SB2	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-
AP1BA1	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYSA1	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	+
TOP1BB2B	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
KVYPSA1	Stem	Kaituna Valley	-	-	-	-	-	-	+++	-	+
KVYPRC2	Root	Kaituna Valley	-	-	-	-	-	-	-	-	-
ROP1RC1S	Root	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
TP2RC1S	Root	Taihape	-	+	-	-	-	-	-	-	-
TOP1RA1S	Root	Tongariro Nat. Park	-	-	-	-	+	+	+++	-	+++
ROP2SC1B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
KIP2RA2S	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
KIP2RA1S	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
TOYRB1S	Root	Tongariro Nat. Park	-	+	-	-	-	-	-	-	+
KIP2RA2B	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
TOYPRA2B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOYPRB3B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TP2RB1R	Root	Taihape	-	-	-	-	-	-	-	-	-
TOYPRC2B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	+
ROP1SC1B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
KIP2RB2B	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
KIP2RB21B	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
TOP1SC2R	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	+
KIP2SB2B	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
TP1RC4B	Root	Taihape	+	++	-	+	-	-	-	-	-

Table B5 continued

KIP2RA3R	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-	
TOP1SC3B	Stem	Tongariro Nat. Park	-	+	-	-	-	-	-	-	-	
APYRA1	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-	
APYRC1	Root	Arthurs Pass	-	+	+	+	+	+	-	-	+++	
APYRC3	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-	
AP1SC2	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-	
AP1SC1	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-	
AP1BB2	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-	
AP1BB1	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-	
APYRB1	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-	
APYRA1FB	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-	
APYRC2	Root	Arthurs Pass	-	-	+	-	-	-	-	-	-	
ROP2SB1	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	-	
KIP2RB1S	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	-	
KIP2RB1BS	Stem	Kaimanawa Forest	-	-	+	+	+	+	-	-	+++	
KIP2RB3B	Stem	Kaimanawa Forest	-	-	-	+	-	-	-	-	-	
TOYPSB1R	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-	
TP1BC2R	Root	Taihape	-	-	-	-	-	-	-	-	-	
TP1SA1R	Stem	Taihape	-	-	-	-	-	-	-	-	-	
TP1SB1R	Stem	Taihape	+	+	-	+	-	-	-	-	-	
TP1SA2R	Stem	Taihape	-	-	-	-	-	-	-	-	+	
ROP1RB2R	Root	Lake Rotopounamu	-	-	-	-	-	-	-	-	-	
TOYP1RB3R	Root	Tongariro Nat. Park	-	+	-	-	-	-	-	-	-	
TP1BA1R	Stem	Taihape	-	-	-	-	-	-	-	-	-	
KRP1BA1	Stem	Kahurangi Nat. Park	+++	+++	++	-	-	-	-	-	+	
KIP2BA1B	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	-	
TOP1RC2B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-	
TP2SC1B	Stem	Taihape	-	-	-	-	-	-	-	-	-	

Table B5 continued

ROP2SA2B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
KRP1BB3	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	-
KRP1BB3Y	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	-
KRP1SC1	Stem	Kahurangi Nat. Park	+++	+++	+++	+++	-	-	+++	-	+++
APYRB2	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYBB1	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYRC1FB	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYRC12X	Root	Arthurs Pass	+	-	-	-	-	-	-	-	-
APYRC11X	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYRC11S	Root	Arthurs Pass	-	++	-	-	-	-	-	-	+++
APYRA12S	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYRA11X	Root	Arthurs Pass	-	-	-	-	-	-	-	-	-
APYSA12X	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	+
APYSA11X	Stem	Arthurs Pass	+	+	-	-	-	-	-	-	+
KVYPRA2	Root	Kaituna Valley	-	+	-	+	-	-	-	-	+
KVYPSA1	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	+
KVYPRA1	Root	Kaituna Valley	-	-	-	-	-	-	-	-	-
APYLC1	Leaf	Arthurs Pass	-	-	-	-	-	-	-	-	-
KRP1BC1	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	++
KVYPBA2	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	++
KVYPBA1	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	-
KRP1SC11	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	+
TOP1BB1	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1SCB1	Stem	Tongariro Nat. Park	++	++	+	+	-	-	-	-	+++
KRP1SC12	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RB12	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RB11	Root	Tongariro Nat. Park	-	-	-	-	-	+	-	-	++
TOP1RC11	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-

Table B5 continued

TOP1RC12	Root	Tongariro Nat. Park	-	++	+	+	-	-	-	-	+++
TOP1RA22	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RA21	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RC21	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RC22	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
KIP2RA4B	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
KVYPRA1	Root	Kaituna Valley	-	-	-	-	-	-	-	-	-
TP2RA2	Root	Taihape	-	-	-	-	-	-	-	-	-
TP2RA1	Root	Taihape	-	++	+	+	-	-	-	-	+
KIP2RB1R	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
KIP2RA1R	Root	Kaimanawa Forest	-	-	-	-	+	-	-	-	-
TP1RC1R	Root	Taihape	++	++	-	-	-	-	-	-	-
TP1RC3B	Root	Taihape	-	-	-	-	-	-	-	-	-
TP1RC2B	Root	Taihape	-	-	-	-	-	-	-	-	+
TP1SC1B	Stem	Taihape	-	-	-	-	-	-	-	-	-
TOP1SC1	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1BC1B	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1SC2	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
AP1SA1	Stem	Arthurs Pass	-	-	-	-	++	++	+++	+++	+++
KRP1BC2	Stem	Kahurangi Nat. Park	-	++	-	-	-	-	-	-	-
KIP1RA11	Root	Kaimanawa Forest	-	++	-	-	-	-	-	-	-
KIP1RA12	Root	Kaimanawa Forest	-	++	-	-	-	-	-	-	-
TOP1SC21	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RB21	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RB22R	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TP2RA21	Root	Taihape	-	-	-	-	-	-	-	-	+
TP2RA22	Root	Taihape	-	-	-	-	-	-	-	-	-
TP2LC1R	Leaf	Taihape	-	++	-	-	-	-	-	-	-

Table B5 continued

TOP1RB1B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TP1BC1R	Stem	Taihape	-	-	-	-	-	-	-	-	-
TP1SC1R	Stem	Taihape	-	-	-	-	-	-	-	-	-
KIP2RA1B	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
TOP1LC1R	Leaf	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TP1RB2B	Root	Taihape	-	-	-	-	-	-	-	-	-
TP1RB1B	Root	Taihape	-	-	-	-	-	-	-	-	-
TP2RC22	Root	Taihape	-	-	-	-	-	-	-	-	-
TP2RA4R	Root	Taihape	-	-	-	-	-	-	-	-	-
TP1LA1B	Leaf	Taihape	+	+++	++	++	-	+	+++	-	+++
TP1LC1B	Leaf	Taihape	+	+++	++	++	-	-	+++	-	+++
TP2RA3B	Root	Taihape	-	-	-	-	-	-	-	-	-
TOYPRB1R	Root	Tongariro Nat. Park	+	++	+	+	-	-	+++	-	-
TOYPRC1R	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOYPRB1B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOYPRB2B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOYPRC1B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOYPRA1B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
KIP1RB1B	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
KIP1RB1R	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
KIP2RA2B	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
KIP1SB1B	Stem	Kaimanawa Forest	+	+++	++	++	+	++	+++	+++	+++
KIP2RA2B	Root	Kaimanawa Forest	-	-	-	-	-	-	-	-	-
ROP1RA1R	Root	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
ROP1LA1B	Leaf	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
ROP2SA1B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	++
ROP1RC1B	Root	Lake Rotopounamu	-	-	-	-	-	-	-	-	-
ROP1SC1B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	-

Table B5 continued

TP2RA3R	Root	Taihape	-	-	-	-	-	-	-	-	-
TP2RC1R	Root	Taihape	-	-	-	-	-	-	-	-	++
TOP1LC2B	Leaf	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RA1B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RA2B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1RA1R	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	++
TP2RC3R	Root	Taihape	-	-	-	-	-	-	-	-	-
TOP1SB1R	Stem	Tongariro Nat. Park	-	+++	-	-	-	-	-	-	-
TOP1SA1R	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-
TOP1SC1R	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	++
TP2RB2B	Root	Taihape	-	-	-	-	-	-	-	-	-
PRY2BRC3	Root	Paringa Forest	-	-	-	-	++	-	-	-	++
P3BLC1	Leaf	Peel Forest	-	-	-	-	-	-	-	-	-
PRY1ASA1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PRY2ABB1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
P4BBB1	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
P3SBC2	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
P3SBC1	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
PR1ASB1A	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PRY2BBC1	Stem	Paringa Forest	-	-	-	-	++	-	+++	+	++
PR1BSA1	Stem	Paringa Forest	+	+	-	-	-	-	-	-	-
PRY1SSC1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	++
P2ABA1	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
PRY2BBB1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
P2BBB1	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
P4BBA1	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
P5ASA2B	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
PR1BB1	Stem	Paringa Forest	-	-	-	-	+	+	-	-	-

Table B5 continued

PR2BSA2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	++
PR1SA3	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
P5SA2A	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
P5SA1	Stem	Peel Forest	-	-	-	-	-	-	-	-	++
PRY2SBB1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PRY2BB2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	++
PR2SB1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PR1SC1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PRY3LB1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	++
PRY2RC2	Root	Paringa Forest	-	-	-	-	-	-	-	-	-
P3BLC2	Leaf	Peel Forest	-	-	-	-	-	-	-	-	-
P3BBA2	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
PRY2SBA1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
P2BBB1	Stem	Peel Forest	+	-	-	-	-	-	-	-	-
PRY2BBC1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PR1BSA1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PR1BSB2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
P5ASC1	Stem	Peel Forest	-	-	-	-	-	-	-	-	++
PRY2SBA2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PRY2RB1	Root	Paringa Forest	-	-	-	-	-	-	-	-	-
P3BA1	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
PR1SB1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PR1SC2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PR1SC1	Stem	Paringa Forest	-	+	-	-	-	-	-	-	-
PRY2RA2	Root	Paringa Forest	-	-	-	-	-	-	-	-	-
P5LA1	Leaf	Peel Forest	-	-	-	-	-	-	-	-	+
PRY2BC2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PRY2BC1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-

Table B5 continued

P5SA2A	Stem	Peel Forest	-	-	-	-	-	-	-	-	-	
PR1SA2	Stem	Paringa Forest	-	+	-	-	-	-	-	-	-	
PRY2RA1	Root	Paringa Forest	-	-	-	-	-	-	-	-	+	
TOYPSB1R	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	+	
TP1BC2R	Stem	Taihape	-	-	+	-	-	-	-	-	-	
TP1SA1R	Stem	Taihape	-	-	-	-	-	-	-	-	-	
TP1SB1R	Stem	Taihape	-	-	-	-	-	-	-	-	-	
TP1SA2R	Stem	Taihape	-	-	-	-	-	-	-	-	+	
ROP1RB2R	Root	Lake Rotopounamu	-	-	-	-	-	-	-	-	-	
TOYP1RB3R	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-	
TP1BA1R	Stem	Taihape	-	-	-	-	-	-	-	-	-	
KRP1BA1	Stem	Kahurangi Nat. Park	-	-	-	-	-	-	-	-	+	
KIP2BA1B	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	-	
TOP1RC2B	Root	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-	
TP2SC1B	Stem	Taihape	-	-	-	-	-	-	-	-	-	
ROP2SA2B	Stem	Lake Rotopounamu	-	-	-	-	-	-	-	-	+	
TOYPLA1B	Leaf	Tongariro Nat. Park	-	-	-	-	-	-	-	-	-	
KIP2SA1R	Stem	Kaimanawa Forest	-	-	-	-	-	-	-	-	-	
AP1SB2	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-	
AP1BA1	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	+	
APYSA1	Stem	Arthurs Pass	-	-	-	-	-	-	-	-	-	
TOP1BB2B	Stem	Tongariro Nat. Park	+	++	-	-	-	-	-	-	-	
KVYPSA1	Stem	Kaituna Valley	-	-	-	-	-	-	-	-	-	
TOYPSC1R	Stem	Tongariro Nat. Park	-	-	-	-	-	-	-	-	+	
TP2SR12	Root	Taihape	-	-	-	-	-	-	-	-	-	
PRY2SA1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-	
P3LC3	Leaf	Peel Forest	-	-	-	-	-	-	-	-	-	
P1SA1	Stem	Peel Forest	-	-	-	-	+	-	-	-	-	

Table B5 continued

PRY2BC1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
P5SB1	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
P1BA1	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
PRY2BB2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
PR1SC1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
P5SA2B	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
P4BA1	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
PRY2RB1	Root	Paringa Forest	-	-	-	-	+	-	-	-	+
P2BB1	Stem	Peel Forest	+	+	-	-	-	-	-	-	+
P3BC1	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
PRY2BBA	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PR1BB1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PR2SA2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
P5SB1	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
PR1SA3	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
PRY2BA2	Stem	Paringa Forest	-	-	-	-	+	-	-	-	+
PRY2BB1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
P5SC1	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
PRY2RC3	Root	Paringa Forest	-	-	-	-	-	-	-	-	+
PRY2RB1	Root	Paringa Forest	-	-	-	-	-	-	-	-	-
P4BA1	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
PR1SB2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
P2BA1	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
PR1SB1A	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
PRY1SA1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+++
P5SC1	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
P2BB1	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
P3BC2A	Stem	Peel Forest	-	-	-	-	-	-	-	-	+

Table B5 continued

P3BC2B	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
P3BC1	Stem	Peel Forest	+	-	-	-	-	-	-	-	+
PRY3BA2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
P3LC2	Leaf	Peel Forest	-	-	-	-	-	-	-	-	+
PRY2BA1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
P3BA1	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
P5SA2B	Stem	Peel Forest	-	-	-	-	-	-	-	-	+
PRY2BB2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+++
PRY2RA2	Root	Paringa Forest	-	-	-	-	-	-	-	-	+
PRY2BC1	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
PRY2BC11	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
PR1SA2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
PR2SA2	Stem	Paringa Forest	-	-	-	-	-	-	-	-	+
P3LC2	Leaf	Peel Forest	-	-	-	-	-	-	-	-	+
P4BA2	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
P5SC13	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
P4BB12	Stem	Peel Forest	-	-	-	-	-	-	-	-	-
PRY2BA4	Stem	Paringa Forest	-	-	-	-	-	-	-	-	-
P1RC2	Root	Peel Forest	-	-	-	-	-	-	-	-	-

Appendix C

C.1 Bennett's Agar

	per litre
Beef Extract (Acumedia, Neogen)	1 g
Yeast Extract (Difco)	1 g
Casein enzyme hydrolysate (Difco)	2 g
Glucose (Scharlau, Scharlab S.L)	10 g
Agar (Difco)	15 g

pH adjusted to 7.3 before autoclaving

Amended with Nystatin and Cycloheximide (50 µg/mL)

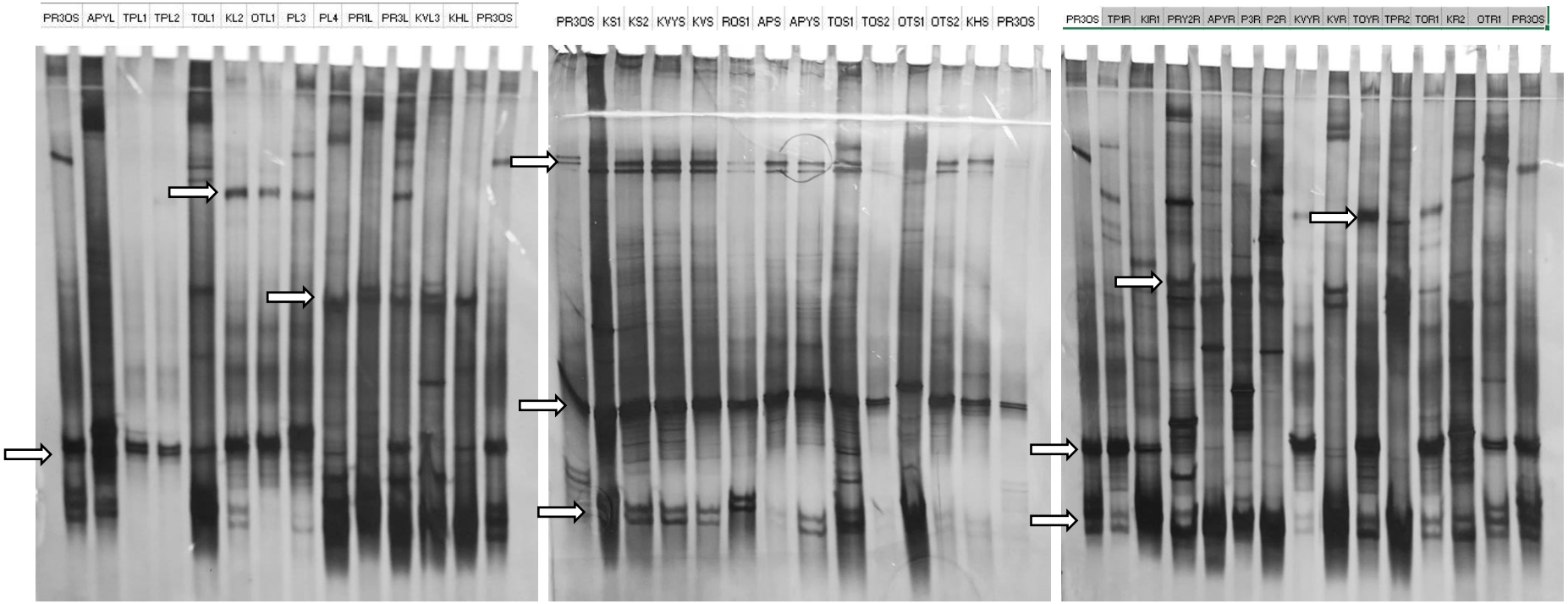
C.2 Starch Casein Agar

	per litre
Soluble Starch (Fischer Chem)	10 g
Casein enzyme hydrolysate (Difco, BD Company)	0.3 g
Sodium Chloride (LabServ, Thermofisher Scientific)	2 g
Potassium Nitrate (Univar, Ajax Fine Chem)	2 g
DiPotassium Hydrogen Phosphate (Labserv)	2 g
Magnesium Sulphate (Scharlau, Scharlab S.L)	0.05 g
Calcium Carbonate (LabServ, Thermofisher Scientific)	0.02 g
Ferrous Sulphate (LabServ, Thermofisher Scientific)	0.01 g
Agar (Difco)	20 g

pH adjusted to 7.2 before autoclaving

Amended with Nystatin and Cycloheximide (50 µg/mL)

C.3 Bands from DGGE gels that were excised, amplified with PCR and sequenced for identifying non-culturable endophytic Actinobacteria



C.4 TriCalcium Phosphate Agar (Frey-Klett *et al.,* 2005)

	per litre
Tricalcium phosphate (Sigma-Aldrich, New Zealand)	4 g
Ammonium chloride (LabServ, Thermofisher Scientific)	5 g
Sodium Chloride (LabServ, Thermofisher Scientific)	1 g
Glucose (Scharlau, Scharlab S.L)	10 g
Magnesium sulphate (Scharlau, Scharlab S.L)	1 g
Agar (Difco, BD Company)	20 g

pH adjusted to 7.2 before autoclaving

C.5 Bands identified as chloroplasts from DGGE based on 16S rRNA sequencing

DGGE band	Closest match	Accession no.	% identity
2L2B	*Peucedanum japonicum chloroplast*	KU866530.1	100
2L7B	*Peucedanum japonicum chloroplast*	KU866530.1	100
1L4A	*Peucedanum japonicum chloroplast*	KU866530.1	100
1L5A	*Peucedanum japonicum chloroplast*	KU866530.1	100
1L5B	*Peucedanum japonicum chloroplast*	KU866530.1	100

Appendix D

D. 1 ANOVA result of the effect of endophytic Actinobacteria and bacteria on the mean shoot length, Shoot dry weight, Root dry weight and number of internodes of *P. colorata* seedlings

Shoot Heights					
Source	DF	SS	MS	F- Value	P-Value
Treatment	4	46.65	11.664	3.42	0.016
Error	45	153.63	3.414		
Total	49	200.28			

Shoot Dry Weight					
Source	DF	SS	MS	F- Value	P-Value
Bacteria and Actinobacteria	4	2.897	0.7243	6.99	0.000
Error	45	4.666	0.1037		
Total	49	7.563			

Root Dry Weight					
Source	DF	SS	MS	F- Value	P-Value
Treatment	4	0.6128	0.15320	4.05	0.007
Error	45	1.704	0.03787		
Total	49	2.3168			

Number of internodes					
Source	DF	SS	MS	F- Value	P-Value
Treatment	4	74.28	18.57	42.85	0.000
Error	45	19.5	0.4333		
Total	49	93.78			

D.2 ANOVA result of the effect of endophytic Fungi on the mean shoot length, root weight, shoot weight and internodes of *P. colorata* seedlings

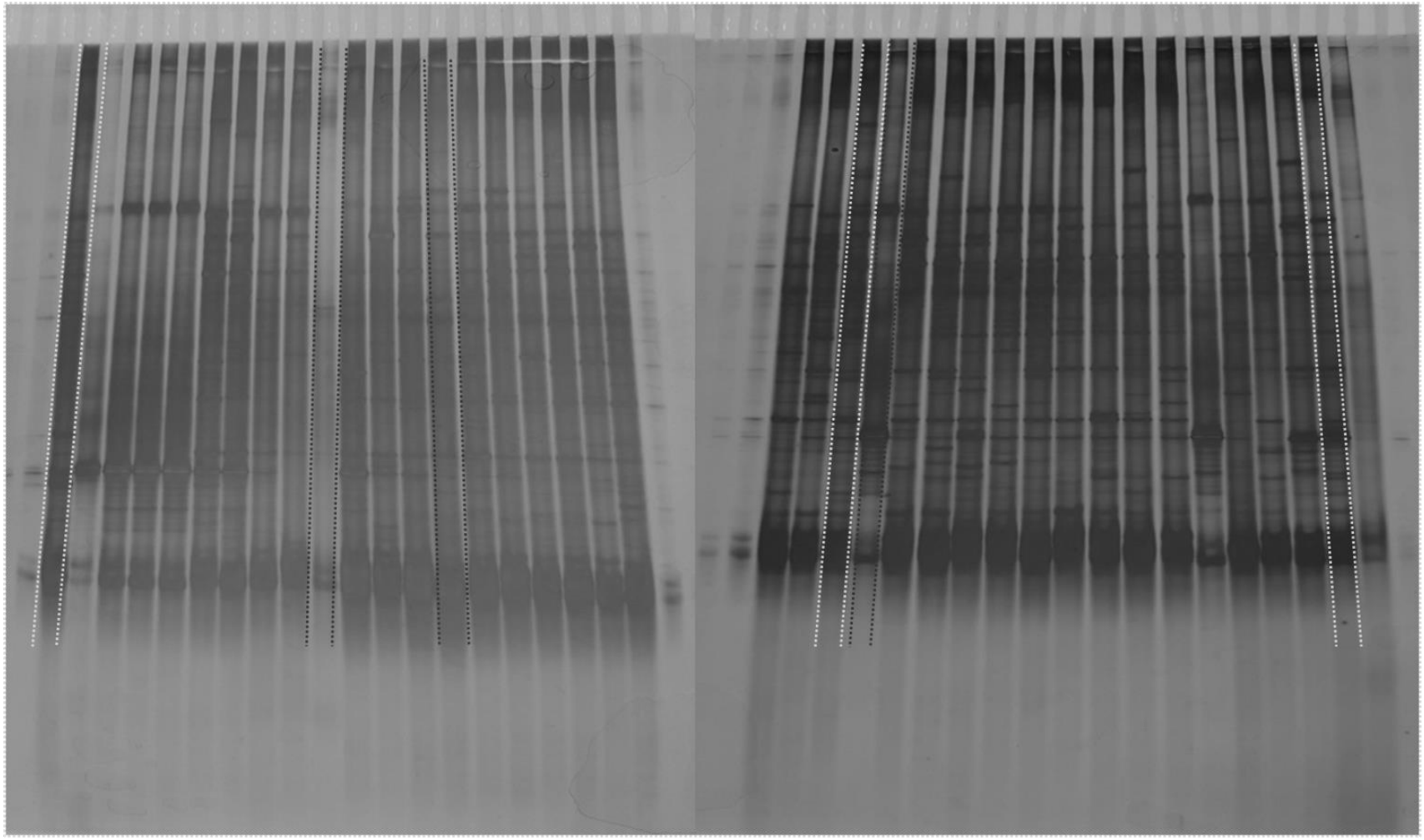
Fungi Shoot heights					
Source	DF	SS	MS	F- Value	P-Value
Treatment	7	155.6	22.227	5.63	0.000
Error	72	284.2	3.947		
Total	79	439.8			

Fungi Root weight					
Source	DF	SS	MS	F- Value	P-Value
Treatment	7	0.4875	0.06964	1.20	0.314
Error	72	4.18	0.05806		
Total	79	4.6675			

Fungi Shoot Weight					
Source	DF	SS	MS	F- Value	P-Value
Treatment	7	0.4239	0.06055	0.42	0.888
Error	72	10.431	0.14488		
Total	79	10.8549			

Number of internodes fungi					
Source	DF	SS	MS	F- Value	P-Value
Treatment	7	67.09	9.5839	11.44	0.000
Error	72	60.3	0.8375		
Total	79	127.39			

D.3 DGGE gel showing Actinobacteria communities in the roots of *P. colorata* seedlings; white dotted lines indicate treatments with *Bacillus* sp. TP1LA1B and black dotted lines indicate treatments with *Metarhizium* sp. PR1SB1.



D. 4 Mean number of bands representing total fungi in the roots of *P. colorata* seedlings treated with endophytic inoculants

Treatment	Average number of bands (n=3)
AP1SA1	18
Control	16
P4BB2	14
P4LA3	13
P4LC2	16
PR1BC2	13
PR1SB1	15
PRY2BA21	14
PRY3BC1	23
TP1BA1B	18
TP1LA1B	18
UKCW B	13

D.5 NMR analysis of 10 *P. colorata* seedlings randomly selected from the seedling lot to identify total dialdehydes and relative ratio of polygodial and 9-deoxymuzigadial.

Plant	Dried leaf mass extracted (mg)	Relative total dialdehydes*	Dialdehyde ratio P:D[#]
1	50	39	10 : 9
2	50	96	10 : 2
3	50	51	10 : 5
4	50	91	10 : 2
5	39	47	10 : 3
6	50	52	10 : 13
7	50	58	10 : 3
8	48	54	10 : 4
9	50	22	10 : 5
10	50	48	10 : 3
Mean (std dev)		56 (22)	10 : 5

*Relative to DMF internal standard and corrected for mass, [#]Polygodial : 9-Deoxymuzigadial